Alveolar Macrophages from Overweight/Obese Subjects with Asthma Demonstrate a Proinflammatory Phenotype

Njira L. Lugogo[1], John W. Hollingsworth[1,2], Druhan L. Howell[3], Loretta G. Que[1], Dave Francisco[1], Tony D. Church[1], Erin N. Potts-Kant[1], Jennifer L. Ingram[1], Ying Wang[1], Sin-Ho Jung[4], and Monica Kraft[1]

[1]Department of Medicine and [2]Department of Immunology, Duke University Medical Center, Durham, North Carolina; [3]Department of Medicine and Department of Pediatrics, University of South Alabama College of Medicine, Mobile, Alabama; and [4]Department of Biostatistics and Bioinformatics, Duke University Medical Center, Durham, North Carolina

Rationale: Obesity is associated with increased prevalence and severity of asthma. Adipose tissue macrophages can contribute to the systemic proinflammatory state associated with obesity. However, it remains unknown whether alveolar macrophages have a unique phenotype in overweight/obese patients with asthma.
Objectives: We hypothesized that leptin levels would be increased in the bronchoalveolar lavage fluid from overweight/obese subjects and, furthermore, that leptin would alter the response of alveolar macrophages to bacterial LPS.
Methods: Forty-two subjects with asthma and 46 healthy control subjects underwent research bronchoscopy. Bronchoalveolar lavage fluid from 66 was analyzed for the level of cellular inflammation, cytokines, and soluble leptin. Cultured primary macrophages from 22 subjects were exposed to LPS, leptin, or leptin plus LPS. Cytokines were measured in the supernatants.
Measurements and Main Results: Leptin levels were increased in overweight/obese subjects, regardless of asthma status ($P = 0.013$), but were significantly higher in overweight/obese subjects with asthma. Observed levels of tumor necrosis factor-α were highest in overweight/obese subjects with asthma. *Ex vivo* studies of primary alveolar macrophages indicated that the response to LPS was most robust in alveolar macrophages from overweight/obese subjects with asthma and that preexposure to high-dose leptin enhanced the proinflammatory response. Leptin alone was sufficient to induce production of proinflammatory cytokines from macrophages derived from overweight/obese subjects with asthma.
Conclusions: *Ex vivo* studies indicate that alveolar macrophages derived from overweight/obese subjects with asthma are uniquely sensitive to leptin. This macrophage phenotype, in the context of higher levels of soluble leptin, may contribute to the pathogenesis of airway disease associated with obesity.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Obesity is associated with an increased risk of developing asthma, and obese patients with asthma have more severe disease with altered response to standard therapies.

What This Study Adds to the Field

We show that, similar to adipocyte tissue macrophages that exhibit an increased proinflammatory response in obesity, primary alveolar macrophages derived from overweight/obese subjects with asthma demonstrate enhanced proinflammatory responses. Our data suggest an interaction between asthma and obesity, which results in a unique population of alveolar macrophages with a proinflammatory response to leptin stimulation.

Keywords: tumor necrosis factor-α; leptin; innate immunity; lipopolysaccharide; environmental lung disease

There is an increased risk of developing asthma in individuals who are overweight and obese (subsequently referred to as overweight/obese) (1–5). The risk of asthma increases steadily with increasing body mass index (BMI) and is higher in women (1, 6). Furthermore, BMI negatively impacts asthma quality of life scores, asthma control scores, severity of exacerbations, and asthma-related hospitalizations (6–9). Together, these studies suggest a role of obesity in the pathogenesis of asthma. However, the mechanisms by which obesity may contribute to airway disease remain poorly understood.

Evidence suggests that adipose tissue is metabolically active, participating not only in energy homeostasis but also in inflammation (12). Adipose tissue secretes biologically active cytokines, including tumor necrosis factor (TNF)-α and IL-6, as well as adipokines, including leptin, adiponectin, plasminogen activator inhibitor (PAI)-1, and resistin (10, 11). Obesity is associated with systemic inflammation, which is characterized by elevated serum levels of these adipokines, chemokines, and acute-phase proteins (12). Adipose tissue is infiltrated by a significant number of macrophages that contribute to inflammation (13). These adipose tissue macrophages can secrete TNF-α, which contributes to insulin resistance in overweight/obese individuals (14). It remains unclear whether obesity is associated with an altered macrophage phenotype in the lung.

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Correspondence and requests for reprints should be addressed to Njira Lugogo, M.D., Duke University Medical Center, P.O. Box 2629, Durham, NC 27710. E-mail: njira.lugogo@duke.edu

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Obesity is also associated with altered levels of serum leptin, and the effects of leptin are believed to be proinflammatory in diseases outside of the lung (15). Leptin is associated with increased airway hyperresponsiveness and serum IgE in murine models (16). In humans, higher serum leptin levels are associated with the presence of asthma, an effect that is more prominent in women. However, serum leptin levels do not entirely account for the increased risk of asthma associated with obesity (17). Leptin levels are elevated in bronchoalveolar lavage fluid (BALF) obtained from overweight/obese subjects and is correlated to increased BMI. BALF leptin content is correlated with serum leptin content, leading to the conclusion that leptin diffuses into the lung from the circulation (18). Interestingly, overweight/obese individuals with asthma demonstrated the highest levels of both BALF and serum leptin. However, no differences in inflammatory markers or oxidative stress were noted in this cohort (18). In spite of these preliminary findings the role of leptin in the pathogenesis of airway disease remains poorly understood.

The aim of this study was to determine whether the level of leptin in the airspace was associated with either BMI or lung function, and whether leptin contributes to the pathobiology of asthma in overweight/obese subjects. Inclusion of both overweight/obese and lean asthmatic and nonasthmatic (normal control) subjects facilitated identification of a unique macrophage phenotype dependent on both asthma and obesity.

Some of the results of these studies have been previously reported in the form of an abstract (19).

METHODS

Study Subjects

The Duke University (Durham, NC) Institutional Review Board approved the protocol. Subjects were recruited from the population in Durham County and the surrounding areas. Informed consent was obtained from each subject (18–65 yr of age). Obese (BMI, >30) and overweight (BMI, 25–30) subjects were pooled into one group denoted as "OA" for overweight/obese asthma and "ON" for normal control subjects (20). Subjects with asthma met National Asthma Education and Prevention Program criteria for mild asthma: had physician-diagnosed asthma, the presence of reversible airflow obstruction, and a methacholine PC_{20} (provocative concentration of methacholine causing a 20% fall in FEV_1) not greater than 8 mg/ml; and used only rescue bronchodilators. Healthy subjects had a methacholine PC_{20} greater than 16 mg/ml, no evidence of airflow obstruction, and no history of pulmonary disease. Exclusion criteria included use of maintenance medication for treatment of asthma, antibiotics, or oral corticosteroids within 4 weeks of the study. In general, subjects who used inhaled corticosteroids within 4 weeks of study enrollment were excluded.

Protocol

Bronchoscopy, bronchoalveolar lavage fluid measurements, and ELISA. Research bronchoscopy was performed on 88 subjects and cell counts/differentials were determined as previously described (21). BALF from 66 subjects was concentrated 20-fold (Millipore, Billerica, MA). Adiponectin and leptin were quantified by ELISA (R&D Systems, Minneapolis, MN). A multiplex cytokine assay was used to measure IL-1β, IL-5, IL-6, IL-8, IL-10, IL-12 (p70), IL-13, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1β, IFN-γ–inducible protein-10 (IP10), vascular endothelial growth factor (VEGF), and TNF-α (Millipore, Billerica, MA). BALF protein levels were analyzed (Pierce, Rockford, IL). Cytokine levels were then normalized to protein and recorded as picograms per milligram.

Macrophage Ob-Rb receptor expression and functional response. Reverse transcription-polymerase chain reaction (RT-PCR) was used to determine expression of the long form of the leptin receptor (Ob-Rb) (Hs00174497_m1 [cat. no. 4331182]; Applied Biosystems/Life Technologies, Carlsbad, CA). *Ex vivo* macrophage experiments were performed on samples obtained from 22 subjects. Briefly, 5×10^5 cells per well were cultured for 2 hours, and nonadherent cells were removed. Cells were cultured in triplicate in the presence/absence of leptin (250 ng/ml, 20 h) followed by ultrapure *Escherichia coli* 0111:B4 LPS (InvivoGen, Carlsbad, CA) (100 ng/ml, 90 min). Dose and duration of LPS exposure were based on studies with macrophages obtained from overweight subjects with asthma (*see* Figure E1 in the online supplement) and prior publications that used leptin at 500 ng/ml in experiments assessing the peritoneal macrophage response to LPS (22). The cell-free supernatants were stored at –80°C and cells were stored in TRIzol (Invitrogen, Carlsbad, CA). Multiplex ELISA (Millipore, Billerica, MA) was used to measure cytokine levels (IL-5, IL-6, IL-8, IL-10, IFN-γ, and TNF-α) in supernatants obtained from these experiments.

Statistical Analysis

Statistical analyses were performed with JMP statistical software (SAS, Cary, NC). The distribution of cytokine levels was not a normal Gaussian distribution and therefore the data were log-transformed with base 10. Two-way analysis of variance (ANOVA) with an interaction term was used to compare demographics and cytokine and adipokine levels. Cell counts, BALF leptin, adiponectin, and cytokine levels were dependent variables; sex, weight, and asthma status were independent variables. We performed analyses for two- and three-way interactions using the independent variables. We adjusted the analysis for BMI. We also adjusted for multiple comparisons using the Bonferroni method with familywise error rate controlled at 10% and an unadjusted P value less than 0.003 was required to achieve significance.

To further characterize the differences in BALF leptin levels, BALF TNF-α levels, and Ob-Rb expression, we performed one-way ANOVA with nonparametric Wilcoxon rank sum test and Tukey-Kramer test for group comparisons.

Linear regression was used to determine the relationship between log-transformed normalized BALF leptin and log BMI and FEV_1. Two-way ANOVA was used to determine the effects of BALF leptin levels on FEV_1 after adjusting for BMI. Methacholine PC_{20} was not normally distributed and therefore Spearman's ρ was used to determine the correlation between methacholine PC_{20} and BALF leptin levels.

The cytokine levels obtained from macrophage culture supernatants were normalized to unexposed control and analyzed by one-way ANOVA with paired comparisons. Paired t tests were used to compare the pretreatment and posttreatment cytokine levels within each group as data were normally distributed. Two-way ANOVA was used to determine the effects of weight status, asthma status, and a combination of these factors on the production of various cytokines in macrophage supernatants. Statistical significance was defined as two-sided $P < 0.05$ for all analyses.

RESULTS

Subject Characteristics

Subject characteristics are shown in Table 1. Of the 88 subjects enrolled, 42 had asthma and 46 were normal control subjects. Obesity was associated with an older age group regardless of asthma status ($P = 0.004$). There were no differences in sex between groups. Overweight/obese subjects were more likely to be black ($P = 0.0012$). The mean BMI was not significantly affected by asthma status. Lung function as quantified by FEV_1 (liters) was lowest in the overweight/obese subjects with asthma ($P = 0.022$ compared with lean asthma; $P = 0.034$ compared with obese normal). No subjects took statins or thiazolidinediones, which could have a potential antiinflammatory effect (23–25). Twenty-two subjects were included in the *ex vivo* experiments and the characteristics of this cohort are included

TABLE 1. CLINICAL CHARACTERISTICS OF STUDY SUBJECTS INCLUDED IN ANALYSIS

			Normal Subjects		Subjects with Asthma	
	Weight/Asthma Status	*P* Value	Lean ($n = 23$) Mean (SD)	Overweight/Obese ($n = 23$) Mean (SD)	Lean ($n = 17$) Mean (SD)	Overweight/Obese ($n = 25$) Mean (SD)
Age, yr	**O**	**0.004**	26.9 (6.6)	33.2 (11.1)	25.82 (8.6)	32.8 (12.1)
	A	0.87				
	O–A	0.71				
Sex, male:female	O	0.55	10M:13F	7M:16F	6M:11F	9M:16F
	A	0.92				
	O–A	0.51				
Race	**O**	**0.0012**	16 White	8 White	13 White	16 White
	A	0.39	5 Black	15 Black	2 Black	9 Black
	O–A	0.99	2 Asian		2 Asian	
BMI, kg/m^2	**O**	**0.0001**	22.8 (1.4)	32.4 (4.4)	21.4 (1.86)	33.9 (9.4)
	A	0.61				
	O–A	0.10				
FEV_1, L	**O**	**0.034**	3.69 (0.76)	3.24 (0.64)	3.21 (0.77)	2.97 (0.82)
	A	**0.022**				
	O–A	0.54				
FEV_1, %	O	0.22	102.2 (14.0)	98.4 (9.1)	88.7 (15.6)	85.3 (15.1)
	A	**<0.0001**				
	O–A	0.93				
FEV_1/FVC ratio	O	0.39	82.4	79.8	74	73.7
	A	**<0.0001**				
	O–A	0.54				
Methacholine PC_{20}	O	0.81	>16	>16	0.97 (1.4)	0.99 (1.7)
	A	**<0.001**				
	O–A	0.72				
Number of subjects with allergic rhinitis	O	0.48	3/23	5/23	17/17	22/25
	A	**<0.0001**				
	O–A	0.12				
Number of subjects receiving ICS	O	N/A	N/A	N/A	0/17	1/25
	A	N/A				
	O–A	N/A				
Number of subjects with GERD	O	0.11	1/23	3/23	2/17	7/25
	A	0.19				
	O–A	0.88				

Definition of abbreviations: BMI = body mass index; F = female; GERD = gastroesophageal reflux disease; ICS = inhaled corticosteroids; M = male; N/A = not applicable; methacholine PC_{20} = provocative concentration of methacholine causing a 20% fall in FEV_1.

Variables included in the analysis include weight (O) and asthma (A) with an interaction term (O–A). Bold type represents statistical significance.

in Table 2. The subjects for *ex vivo* experiments were relatively well matched in the four groups except for the lean asthma group, which was composed only of female subjects.

Effects of Obesity on BALF Differential Cell Counts

To determine the effects of obesity on asthma phenotype, we obtained BALF and performed differential cell counts. No group differences were appreciated in BALF differentials (Table E1). In this cohort, 13% of lean subjects with asthma and 15% of overweight/obese subjects with asthma had greater than 2% eosinophils in BALF (data not shown).

Effect of Obesity and Asthma on Adipokine Levels in BALF

Previous work has shown increased levels of leptin in both serum and BALF from overweight/obese subjects (17, 18, 26). We also identified a significant correlation between log BALF leptin levels and log BMI (Figure E2). In our cohort, BALF leptin levels were significantly higher in overweight/obese subjects with asthma (Figure 1A), an effect that was more prominent in females (Figure 1B). There were no detectable differences in leptin levels between groups of male subjects (Figure 1C). Despite the association between adiponectin and asthma (27), we did not identify any differences in BALF adiponectin levels between groups. There were no significant differences in the mean BMI of male and female subjects (26.2 ± 0.88 vs. 28.78 ± 1.16, $P = 0.15$, respectively), suggesting that sex differences in BALF leptin levels are not related primarily to higher BMI in females. In spite of this, the range of BMI values appeared quite different between males and females (21.32–38.87 in males and 18.0–60.9 in females).

We noted an inverse correlation between FEV_1 and log BALF leptin levels ($r^2 = 0.17$, $P = 0.005$) that was present after adjusting for BMI. We did not observe a significant correlation between leptin levels and bronchial hyperresponsiveness.

Effect of Obesity and Asthma on BALF Cytokine Profiles

Previous work supports the hypothesis that a systemic proinflammatory phenotype exists in obesity. Therefore, we measured the levels of IL-1β, IL-5, IL-6, IL-8, IL-10, IL-12 (p70), IL-13, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1β, IP10, VEGF, and TNF-α in BALF. Using a Bonferroni correction, a significant P value in this analysis is considered $P < 0.003$. Although we did not observe significant differences in many of these factors in the BALF, we did observe several trends. The presence of asthma had an effect on BALF IL-5 ($P = 0.011$) and IL-10 ($P = 0.04$) levels. The interaction between asthma and weight status resulted in higher BALF IFN-γ ($P = 0.02$) and TNF-α ($P = 0.03$) levels in overweight/obese subjects with asthma. There was an effect of the sex × weight interaction on MCP-1 levels,

TABLE 2. CHARACTERISTICS OF SUBJECTS INCLUDED IN MACROPHAGE EXPERIMENTS

			Normal Subjects		Subjects with Asthma	
	Weight/Asthma Status	*P* Value	Lean ($n = 5$) Mean (SD)	Overweight/Obese ($n = 4$) Mean (SD)	Lean ($n = 5$) Mean (SD)	Overweight/Obese ($n = 8$) Mean (SD)
Age, yr	O	0.53	28.6 (8.6)	28 (2.0)	24.33 (2.5)	30 (10.6)
	A	0.78				
	O–A	0.44				
Sex, male:female	O	0.95	2M:3F	1M:3F	0M:5F	3M:5F
	A	0.95				
	O–A	0.94				
Race	O	0.97	4 White	2 White	3 White	3 White
	A	0.98	1 Asian	2 Asian	2 Black	5 Black
	O–A	0.98				
BMI, kg/m^2	O	**0.0004**	22.6 (1.4)	29.94 (3.7)	20.8 (1.21)	35.1 (8.6)
	A	0.51				
	O–A	0.18				
FEV_1, L	O	0.74	3.61 (0.76)	3.41 (0.2)	3.1 (0.48)	3.13 (0.56)
	A	0.12				
	O–A	0.64				
FEV_1, %	O	0.82	102.3 (12.7)	101.5 (9.1)	93.3 (6.12)	93.4 (10.5)
	A	0.62				
	O–A	0.68				
FEV_1/FVC ratio	O	0.61	88.9	84.2	76.7	78.3
	A	**<0.008**				
	O–A	0.31				
Methacholine PC_{20}	O	0.14	>16	>16	3.02 (2.7)	1.1 (0.9)
	A	**<0.001**				
	O–A	0.14				

Definition of abbreviations: BMI = body mass index; methacholine PC_{20} = provocative concentration of methacholine causing a 20% fall in FEV_1. Variables included in the analysis include weight (O) and asthma (A) with an interaction term (O–A). Bold type represents statistical significance.

with overweight/obese females with asthma having the highest levels of MCP-1 in the BALF ($P = 0.04$). Interestingly, VEGF levels were low in overweight/obese subjects regardless of asthma status ($P = 0.049$). Obesity and female sex had effects on BALF leptin levels ($P = 0.011$ and $P < 0.0001$, respectively) (Table 3).

Prior studies demonstrate that leptin can increase TNF-α production in adipocyte macrophages (13); however, the effects of leptin on lung macrophages are unknown. We aimed to determine whether BALF TNF-α levels differed between groups and noted higher levels in overweight/obese subjects with asthma in comparison with lean subjects with asthma ($P = 0.03$) and overweight/obese normal subjects ($P = 0.04$) (Figure 2A), an effect that was more prominent in females (Figure 2B). There were no observed differences between groups of male subjects (Figure 2C). Furthermore, we determined that BALF leptin and TNF-α are significantly correlated, but in female subjects only ($r^2 = 0.36$, $P = 0.0008$) (Figures 3A–3C).

Leptin Ob-Rb Receptor Expression on Alveolar Macrophages

It is recognized that the Ob-Rb receptor binds the long form of leptin and is the dominant receptor that mediates intracellular signaling. We determined that Ob-Rb is expressed in alveolar macrophages; however, no significant differences in Ob-Rb mRNA expression were noted between the four groups (data not shown). This finding does not necessarily address the functional consequences of soluble leptin on alveolar macrophages.

Functional Response of Alveolar Macrophages in a Manner Dependent on Obesity and Asthma

To determine whether the LPS and leptin response in macrophages is different, based on the presence or absence of asthma and obesity, we exposed primary alveolar macrophages to bacterial LPS *ex vivo*. For the purpose of this article, we have focused on the biological response to the bacterial toxin LPS as a marker of innate immune recognition. Similar to LPS, we hypothesized that there would be an immediate recognition of endogenous leptin in lung macrophages resulting in a proinflammatory signaling response.

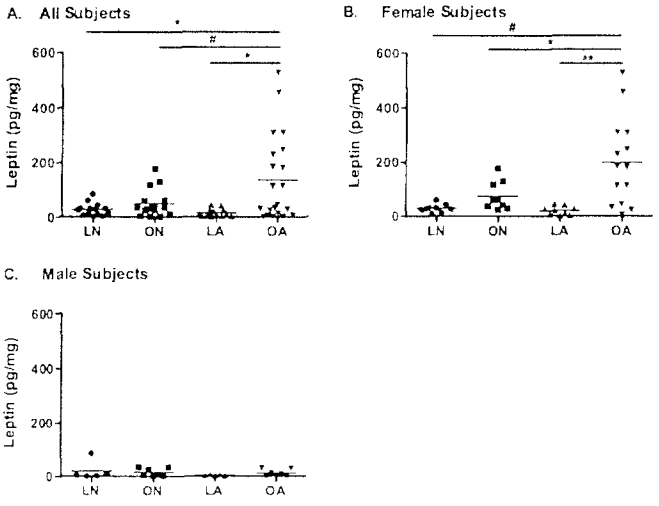

Figure 1. Log-normalized bronchoalveolar lavage fluid (BALF) leptin levels in four groups, divided by sex. (*A*) BAL leptin levels were significantly higher in overweight/obese subjects with asthma and in (*B*) overweight/obese females with asthma. (*C*) BALF leptin levels do not differ in males. LA = lean subjects with asthma; LN = lean normal subjects; OA = overweight/obese subjects with asthma; ON = overweight/obese normal subjects. $^{**}P < 0.001$; $^{*}P < 0.01$; $^{\#}P < 0.05$.

TABLE 3. NORMALIZED BRONCHOALVEOLAR LAVAGE FLUID CYTOKINE LEVELS

			Normal Subjects		Subjects with Asthma	
BAL Biomarker	Weight/Asthma Status	*P* Value	Lean (*n* = 17) Mean (95% CI)	Overweight/Obese (*n* = 17) Mean (95% CI)	Lean (*n* = 12) Mean (95% CI)	Overweight/Obese (*n* = 20) Mean (95% CI)
IFN-γ, pg/ml	O	0.91	8.4 (4.1–17.3)	3.31 (1.5–7.7)	4.70 (2.0–10.7)	10.2 (6.9–15.1)
	A	0.49				
	O–A	**0.02**				
IL-10, pg/ml	O	0.63	2.36 (1.02–5.37)	1.26 (0.59–2.75)	2.52 (0.92–6.98)	3.71 (2.47–5.57)
	A	**0.04**				
	O–A	0.37				
IL-5, pg/ml	O	0.74	0.83 (0.4–1.9)	0.49 (0.2–1.0)	1.03 (0.5–2.2)	1.60 (1.0–2.5)
	A	**0.01**				
	O–A	0.43				
TNF-α, pg/ml	O	0.41	7.03 (2.2–10.9)	4.56 (2.9–7.3)	3.79 (2.0–7.1)	9.82 (6.6–14.4)
	A	0.22				
	O–A	**0.03**				
MCP-1, pg/ml	O	0.63	691.83 (467.7–1,023.3)	724.43 (524.81–1,000)	630.95 (363.1–1,096.5)	616.59 (446.68–851.14)
	A	0.76				
	O–A	0.89				
VEGF, pg/ml	O	**0.05**	1,000 (575.44–1,737.8)	549.5 (371.53–812.83)	1,122 (602.55–2,137.9)	676.08 (416.87–1,122.1)
	A	0.57				
	O–A	0.93				
Leptin, pg/ml	O	**0.011**	26.97 (13.19–40.76)	49.32 (22.1–76.54)	15.35 (5.91–24.79)	142.45 (67.78–217.11)
	A	0.33				
	O–A	0.33				

Definition of abbreviations: BAL = bronchoalveolar lavage; CI = confidence interval; MCP-1 = monocyte chemotactic protein-1; TNF-α = tumor necrosis factor-α; VEGF = vascular endothelial growth factor.

Independent factors include weight (O), asthma (A), and the interaction term (O–A). Bold type represents statistical significance.

We exposed lung macrophages to leptin, LPS, and leptin pretreatment followed by LPS exposure. Macrophages obtained from overweight/obese subjects with asthma produced significantly higher levels of IL-5, IFN-γ, TNF-α, and IL-10 in response to leptin, LPS, and leptin followed by LPS when compared with their respective unexposed control state ($P < 0.05$). We observed no increases in IL-6 production in response to leptin. However, LPS and leptin–LPS exposure resulted in significant increases in IL-6 production in overweight/obese subjects with asthma ($P = 0.047$ and $P = 0.031$, respectively). IL-8 levels were increased in response to both leptin ($P = 0.016$) and leptin–LPS ($P = 0.047$) but not LPS alone. There were no differences in cytokine production in the other three subject groups. Interestingly, only macrophages from overweight/obese subjects with asthma respond in a proinflammatory manner after the 90 minutes of exposure to LPS. This suggests that the response in these macrophages may be the result of secretion of preformed cytokines. The lack of response in the three other subject groups may be related to the duration of LPS exposure.

We then focused on determining whether differential cytokine production occurred between groups of subjects. There was significant variability in baseline cytokine levels and therefore we performed fold change comparisons, as we believe correction for constitutive levels of cytokines most accurately reflects the biological response to leptin and LPS. Leptin increased IL-5 in overweight/obese ($P = 0.006$) and lean subjects with asthma ($P = 0.009$) as compared with lean normal subjects (Figure 4A). IFN-γ levels were significantly higher in overweight/obese subjects with asthma when compared with lean normal ($P = 0.005$) subjects (Figure 4B). In addition, TNF-α and IL-8 levels were higher in overweight/obese subjects with asthma as compared with all the other groups (Figures 4C and 4D). The proinflammatory response to leptin appears most prominent in alveolar macrophages obtained from overweight/obese subjects with asthma.

We then aimed to determine the effects of LPS on lung macrophages in the absence of leptin treatment. LPS significantly increased IL-5 levels in overweight/obese subjects with asthma when compared with overweight/obese ($P = 0.01$) and lean ($P = 0.005$) normal subjects (Figure 4A). There were no differences in IFN-γ (Figure 4B) and TNF-α (Figure 4C) between groups. IL-8 levels were significantly higher in overweight/obese

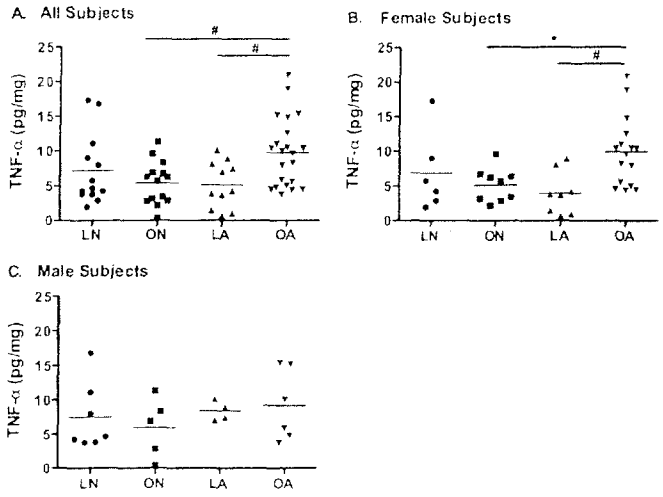

Figure 2. Log-normalized bronchoalveolar lavage fluid (BALF) tumor necrosis factor (TNF)-α levels in four groups, divided by sex. (*A*) BALF TNF-α levels were significantly higher in overweight/obese subjects with asthma and in (*B*) overweight/obese females with asthma. (*C*) BALF leptin and TNF-α levels do not differ in males. LA = lean subjects with asthma; LN = lean normal subjects; OA = overweight/obese subjects with asthma; ON = overweight/obese normal subjects. $^{*}P < 0.01$; $^{\#}P < 0.05$.

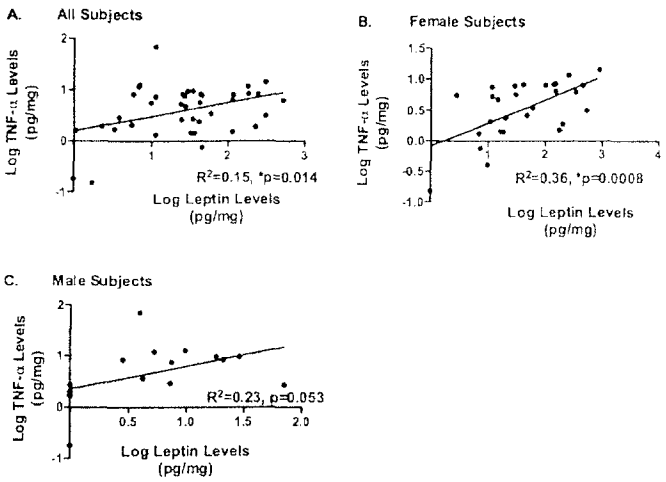

Figure 3. Correlation between bronchoalveolar lavage (BAL) leptin and tumor necrosis factor (TNF)-α in four groups and based on sex. (*A*) BAL TNF-α levels are correlated with BAL leptin levels overall ($R^2 = 0.15$, $^*P = 0.014$). (*B*) There is a significant correlation between BAL TNF-α and leptin in female subjects ($R^2 = 0.36$, $^*P = 0.0008$). (*C*) There is no correlation of these two cytokines in male subjects ($P = 0.053$).

subjects with asthma when compared with lean ($P = 0.01$) and overweight/obese normal subjects ($P = 0.003$) (Figure 4D).

Previous studies provide evidence that leptin can prime subsequent response to LPS in cultured macrophages (22). Therefore, we pretreated macrophages with leptin followed by exposure to LPS. We noted that leptin–LPS exposure significantly increased IL-5 levels in overweight/obese subjects with asthma as compared with the other groups (Figure 4A). In addition, there were significantly higher IFN-γ and TNF-α levels in overweight/obese subjects with asthma when compared with overweight/obese normal ($P = 0.016$ and $P = 0.003$, respectively) and lean asthma ($P = 0.031$ and $P = 0.017$, respectively) subjects (Figures 4B and 4C). IL-8 levels were also higher in overweight/obese subjects with asthma in comparison with lean ($P = 0.013$) and overweight/obese ($P = 0.001$) normal control subjects (Figure 4D). These data indicate that pretreatment of macrophages with leptin before LPS exposure results in an enhanced proinflammatory response and that overweight/obese subjects with asthma have the greatest response. We did not observe lower baseline cytokine levels in overweight/obese subjects with asthma and therefore we attribute the increased fold change in cytokine levels to a more robust response to stimulation with leptin and LPS.

Given the significant response to leptin–LPS exposure noted in overweight/obese subjects with asthma, we aimed to determine whether the effects of leptin and LPS were additive or synergistic in this group of subjects. We noted a significant difference in IL-5 ($P = 0.01$) and TNF-α ($P = 0.02$) levels when comparing leptin and leptin–LPS exposure (Figures 4A and 4C). In addition, we noted a significant difference in IL-5 ($P = 0.02$) and IL-8 ($P = 0.03$) levels in response to leptin–LPS as compared with LPS only (Figures 4A and 4D). The effects of leptin pretreatment followed by LPS exposure appear additive, but we cannot rule out the possibility of leptin priming the macrophage response to LPS.

Last, we performed analyses to determine the effects of weight status, asthma status, and the interaction of these two factors on the difference in cytokine levels after exposure to leptin, LPS, and leptin–LPS. The obesity × asthma interaction resulted in increased IFN-γ, TNF-α, IL-8, and IL-10 in response to LPS. In addition, TNF-α, IL-8, and IL-6 levels were altered by the interaction between obesity and asthma in response to leptin pretreatment followed by LPS exposure (Tables E2A–E2C).

DISCUSSION

This study supports that obesity and asthma intersect to create unique inflammatory responses in the lung. We provide further support for the existing evidence that leptin levels are increased in the lungs of overweight/obese subjects (18, 28) and that female sex impacts the levels of leptin in the lungs. Furthermore, we demonstrate that increased levels of BALF TNF-α

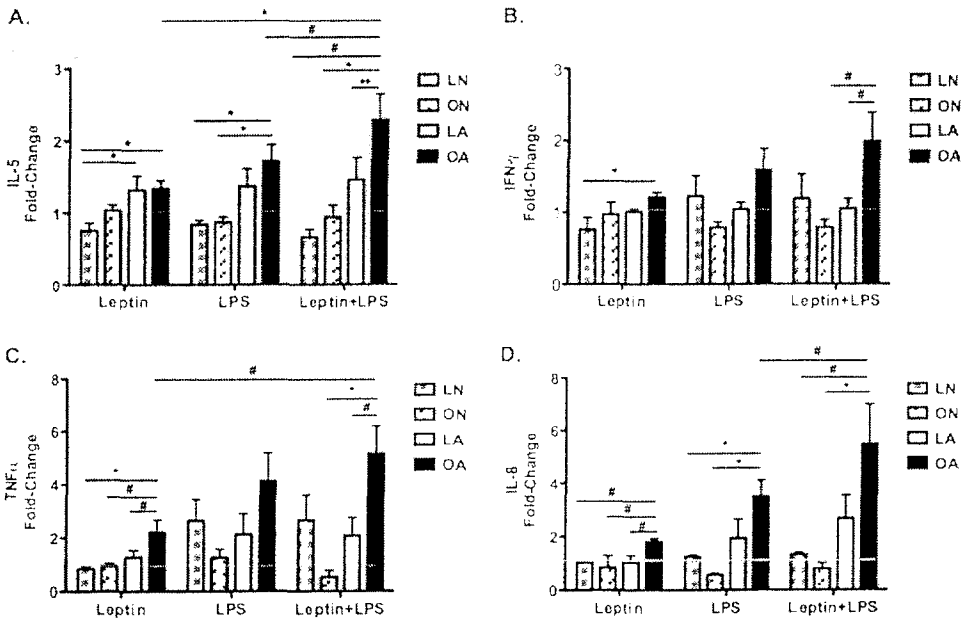

Figure 4. Effect of leptin, LPS, and leptin plus LPS on cytokine production by macrophages from subjects with asthma and normal control subjects. Data are reported as fold change = postexposure cytokine production/negative control. (*A*) Leptin results in higher IL-5 levels in subjects with asthma regardless of weight status. Fold changes in IL-5 levels are higher in overweight/obese subjects with asthma in response to LPS and leptin plus LPS exposure. (*B* and *C*) Tumor necrosis factor (TNF)-α and IFN-γ levels are higher in overweight/obese subjects with asthma in response to leptin. There is no differential response to LPS. Leptin plus LPS results in significantly higher fold changes in IFN-γ and TNF-α levels in overweight/obese subjects with asthma. (*D*) IL-8 levels are higher in overweight/obese subjects with asthma in response to leptin, LPS, and leptin plus LPS. LA = lean subjects with asthma; LN = lean normal subjects; OA = overweight/obese subjects with asthma; ON = overweight/obese normal subjects. $^{**}P < 0.001$; $^*P < 0.01$; $^\#P < 0.05$.

may be related to increased leptin levels in the lungs. The findings in this study demonstrate that overweight/obese subjects with asthma have increased levels of leptin and an increased alveolar macrophage response to high-dose leptin. This microenvironment would be anticipated to generate elevated levels of proinflammatory cytokines in the airspace. It has been previously reported that adipose tissue macrophages exhibit a proinflammatory state (29, 30). Our data provide evidence that alveolar macrophages from overweight/obese subjects with asthma similarly demonstrate a proinflammatory state.

Primary alveolar macrophages derived from overweight/obese subjects with asthma have an enhanced response to high-dose leptin resulting in increased levels of proinflammatory cytokines (IL-8 and TNF-α). The augmented response to leptin is not likely to be related to an increase in Ob-Rb expression, because we observed no differences in receptor expression as determined by mRNA levels. In fact, the augmented response is surprising given the fact that obesity is typically associated with leptin resistance, particularly in the brain (31, 32). The altered response of macrophages may be related to either activation of resident cells or recruitment of a new population of circulating cells. Alternatively, selective leptin resistance may be present in these subjects with central leptin resistance promoting obesity due to lack of appropriate responses to satiety, whereas peripheral cellular responses to leptin remain intact (33). Characterization of macrophage populations derived from the lungs of overweight/obese subjects with asthma could provide improved understanding of this differential response. These observations could have important implications given the leptin-rich environment in the lung and the functional consequences of leptin stimulation on macrophage populations.

This study also demonstrates important interactions between leptin and LPS. Leptin pretreatment resulted in further increases in the levels of IL-5, IFN-γ, and TNF-α in overweight/obese in comparison with lean subjects with asthma. Initially, we hypothesized that leptin primes the subsequent response to LPS as observed in mouse models (22). Our data suggest an additive effect of leptin on the biological response to LPS. Because obesity is associated with increased leptin levels, this could result in increased proinflammatory responses via various environmental stimuli that activate the innate immune system. Increased circulating free fatty acids in overweight/obese subjects could result in increased Toll-like receptor (TLR)-4 expression on macrophages (34), presenting an opportunity for increased LPS binding and the subsequent production of proinflammatory cytokines. Future studies will focus on understanding the distribution of TLRs on macrophages from obese subjects. Interestingly, obesity alone is not sufficient to elicit enhanced proinflammatory response as demonstrated by similar macrophage responses between obese and normal weight individuals without asthma. Overweight/obese subjects with asthma demonstrate an increased macrophage inflammatory response that appears to be a consequence of the interaction between asthma and obesity.

The current study has both strengths and limitations. We have a carefully characterized cohort of subjects with asthma stratified on the basis of BMI. This represents the first report to our knowledge of primary alveolar macrophages from obese subjects with asthma that includes subjects not receiving inhaled corticosteroid therapy. Our study is limited by the use of high doses of leptin, but is similar to previous work performed with murine peritoneal macrophages and response to LPS (22). Of note, serum leptin levels average 75 ng/ml (18). The physiological relevance of our dose of leptin will be determined in future studies. The dose and duration of LPS were based on our kinetic studies of alveolar macrophages obtained from obese/asthmatic subjects. We were surprised by the limited response to leptin and LPS in both the lean normal and obese normal groups. Future studies will require additional doses of leptin and dose–response assessment to LPS. Cell availability limits studies of primary human alveolar macrophages. The effect of atopic status on inflammatory responses and bronchial hyperresponsiveness remains unclear at this time.

Lack of atopy may play a role in alterations in airway hyperresponsiveness in overweight/obese subjects with asthma (35); however, the underlying mechanisms are poorly understood. Our study was underpowered to detect any differences in macrophage responses based on the presence or absence of atopy.

Adipose tissue macrophages play an important role in mediating systemic inflammation and the development of insulin resistance in obesity (36). Macrophages play an important role in the pathogenesis of diseases related to the metabolic syndrome. Prior studies aimed at identifying possible mechanisms that mediate the overweight/obese asthma phenotype have determined that $CD4^+$ T cells do not appear to play a key role in this association (35). We now propose that alterations in macrophage responses create a unique inflammatory environment in the lungs of overweight/obese subjects with asthma. We suspect that macrophages are the key regulatory cells in the lung, but further studies are clearly warranted. It will be crucial to determine whether macrophage responses are altered by significant weight loss. We suggest a paradigm in which adipocyte production of leptin can amplify alveolar macrophage–derived inflammation in the overweight/obese patient with asthma. Our findings provide novel insights into the interaction that exists between obesity and asthma. Continued studies in this area could lead to more effective therapeutic approaches for overweight/obese patients with asthma.

Author disclosures are available with the text of this article at www.atsjournals.org.

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Ambient Ozone Primes Pulmonary Innate Immunity in Mice[1]

John W. Hollingsworth[2,*], Shuichiro Maruoka[†], Zhuowei Li[*], Erin N. Potts[*], David M. Brass[†], Stavros Garantziotis[*,†], Alan Fong[†], W. Michael Foster[*] and David A. Schwartz[†]

[+] Author Affiliations

Abstract

Exposure to ozone in air pollution in urban environments is associated with increases in pulmonary-related hospitalizations and mortality. Because ozone also alters clearance of pulmonary bacterial pathogens, we hypothesized that inhalation of ozone modifies innate immunity in the lung. To address our hypothesis, we exposed C57BL/6J mice to either free air or ozone, and then subsequently challenged with an aerosol of *Escherichia coli* LPS. Pre-exposure to ozone resulted in enhanced airway hyperreactivity, higher concentrations of both total protein and proinflammatory cytokines in lung lavage fluid, enhanced LPS-mediated signaling in lung tissue, and higher concentrations of serum IL-6 following inhalation of LPS. However, pre-exposure to ozone dramatically reduced inflammatory cell accumulation to the lower airways in response to inhaled LPS. The reduced concentration of cells in the lower airways was associated with enhanced apoptosis of both lung macrophages and systemic circulating monocytes. Moreover, both flow cytometry and confocal microscopy indicate that inhaled ozone causes altered distribution of TLR4 on alveolar macrophages and enhanced functional response to endotoxin by macrophages. These observations indicate that ozone exposure increases both the pulmonary and the systemic biologic response to inhaled LPS by priming the innate immune system.

Introduction

Chronic obstructive pulmonary disease (COPD)[3] is the fourth leading cause of death in the U.S., and it is the only common cause of death that is increasing in incidence. Exposure to ambient air pollutants can lead to exacerbations of COPD associated with both enhanced pulmonary inflammation and loss of lung function. Ozone is a common urban air pollutant that significantly contributes to increased pulmonary mortality, especially among those with chronic lung disease (1, 2, 3, 4). It is known that inhalation of ambient ozone can modify adaptive immune function (reviewed in Ref. 5) in both rodents (6, 7, 8, 9) and humans (10, 11). However, COPD is characterized by increased numbers of macrophages, neutrophils, and cytotoxic CD8 lymphocytes, suggesting a role of innate immune function in pathogenesis of this disease. Ozone is known to alter macrophage function (12, 13). Alveolar macrophages exposed to ozone in vitro demonstrate reduced phagocytosis in both rodents (14) and humans (15), which in part is related to cytotoxicity (16). In addition, exposure to ozone is associated with impaired clearance of multiple live organisms, including the following: *Streptococcus zooepidemicus* (17), *Streptococcus pyogenes* (12), *Staphylococcus aureus* (18), *Klebsiella pneumonia* (19), *Mycobacteria tuberculosis* (20), and *Listeria monocytogenes* (21). Altered vulnerability to these bacterial pathogens suggests that ozone could specifically impair innate immune function in the lung and provide an underlying mechanism for the enhanced morbidity and mortality observed in human populations exposed to higher concentrations of ambient ozone.

Understanding the environmental factors that regulate macrophage and neutrophil recruitment and function will improve our understanding of the factors that contribute to the pathogenesis of COPD. To address this possibility, we hypothesized that inhalation of ozone would modify the innate immune response in the lung.

Strict regulation of innate immune function can affect the progression and outcome of several diseases, including bacterial pneumonia, chronic bronchitis, and environmental airways disease. Specific activation of innate immunity in the lung can be achieved by exposure to aerosolized bacterial endotoxin (22). Inhalation of bacterial endotoxin can contribute to the development and progression of occupational lung disease (23). Furthermore, inhaled LPS can modify allergic asthma (24, 25) and cause severe inflammatory disease in both humans and mice (26, 27, 28). Although this immediate inflammatory response plays an important role in host defense, uncontrolled inflammation can contribute to the progression of pulmonary (and systemic) disease (29). Accordingly, it is of considerable interest to identify environmental factors, which may modify innate immune responsiveness.

By focusing on the effect of ozone on innate immunity in the lung, we have found that ambient exposure to ozone can prime both local and systemic innate immune responsiveness by altering the cellular distribution of TLR4 and increased signaling by alveolar macrophages, leading to cellular apoptosis. These findings provide a biological mechanism for the epidemiological relationship between ozone and increased pulmonary morbidity and mortality.

Materials and Methods

Mice

Six- to 8-wk-old male C57BL/6J mice were purchased from The Jackson Laboratory. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Duke University Medical Center and were conducted in accordance with the standards established by the U.S. Animal Welfare Acts.

Exposure protocol

Six- to 8-wk-old male C57BL/6J mice (The Jackson Laboratory) were exposed to either filtered air (FA) or ozone (2 parts per million (ppm) × 3 h), and then challenged with an aerosol of purified 0111:B4 *Escherichia coli* LPS (LPS, 4 $\mu g/m^3$ × 2.5 h), as previously described (22). This dose of LPS models the inflammatory response experienced by grain mill workers during a typical 8-h workday (28, 30). All animals were evaluated 4 h after initiation of the LPS aerosol. The mean level of LPS measured in aerosol was 3.58 $\mu g/m^3$ (2.19–4.96 $\mu g/m^3$), and serum was 0.66 EU/ml (0.28–1.41 EU/ml) by the *limulus* amebocyte lysate assay (BioWhittaker). The level and duration of ozone exposure (2 ppm × 3 h) used in this protocol are an accepted murine model of ozone/oxidant injury in comparison with effective ambient exposure levels in humans. Level of ozone exposure for ex vivo analysis of macrophage function was 1 ppm × 3 h. Ozone or filtered air exposures were performed in 55-L Hinners-style exposure chambers for 3 h. Chamber air at 20–22°C and 50–60% relative humidity was supplied at a rate of 20 changes/hour. O_3 was generated by directing 100% oxygen through a UV light ozone generator. Ozone concentration was monitored continuously within the chamber with an ozone UV light photometer (Dasibi model 1003AH; Dasibi). In vivo experiments represent 10 mice per group with at least two repeats.

Airway physiology

Direct measurements of respiratory mechanics in response to methacholine were made using the flexivent system (Scireq) and reported as total pulmonary resistance (R_T) $cmH_2O/ml/s$. Anesthesia was achieved with 60 mg/kg pentobarbital sodium injected i.p. and ventilated with a computer-

controlled small animal ventilator (flexiVent; Scireq) with a tidal volume of 7.5 ml/kg and a positive end-expiratory pressure of 3 cm H_2O. The mice were then given a neuromuscular blockade (0.8 ml/kg pancuronium bromide). Measurements of respiratory mechanics were made by the forced oscillation technique. Response to aerosolized methacholine (0, 10, 25, and 100 mg/ml) was determined by resistance measurements every 30 s for 5 min, ensuring the parameters calculated had peaked. Total lung capacity breaths were given after each dose, keeping the airways open and returning the measurements back to baseline. The resistance measurements were then averaged at each dose and graphed (R- cmH$_2$O/ml/s) along with the initial baseline measurement.

Lung samples

Whole lung lavage and cell differentials were determined, as previously described (22). Luminex (Bio-Rad) was used to evaluate protein concentrations of keratinocyte cytokine, IL-6, MIP-1α, IL-10, IL-12(p70), and IL-17 with a commercially available immunoassay (Linco Research). Total protein concentrations in lung lavage fluid were determined using the Lowry assay (Bio-Rad).

Chemotaxis assays

Fresh polymorphonuclear cells (PMN) were harvested from either alveolar lavage or peripheral blood from animals exposed to either FA-LPS or ozone-LPS. First, live alveolar neutrophils were harvested from animals exposed to either FA-LPS or ozone-LPS. RBCs were removed with lysis buffer (0.14 M NH_4Cl and 0.015 M Tris (*p*H 7.2)) for 5 min. A total of 1×10^5 cells suspended in RPMI 1640 with 10% FCS was placed in upper Transwell with 5 μm pore size (Costar). Cells were incubated in the presence of leukotriene B_4 (LTB_4; 30 μM) for 1 h at 37 C. Cells were collected from the lower well and stain for neutrophils ($Gr\text{-}1^+$) and quantified by flow cytometry. Second, circulating neutrophils were isolated with Histopaque 1083, per manufacturer's recommendations (Sigma-Aldrich), and analyzed for chemotaxis to LTB_4. Third, lavage supernatants from pre-exposed animals were evaluated for chemotatic factors. A total of 1×10^5 zymosan-elicited peritoneal neutrophils was placed in the upper well, and then 600 μl of supernatants from either FA-LPS or ozone-LPS mice was used as chemoattractant. Neutrophils ($Gr\text{-}1^+$) were quantified in lower well by flow cytometry.

Western blots

Lung tissues were homogenized and lysed in ice-cold lysis buffer containing 20 mM Tris (pH 7.4), 137 mM NaCl, 25 mM β-glycerolphosphate (pH 7.4), 2 mM PPiNa, 2 mM EDTA (pH 7.4), 1% Triton X-100, 10% glycerol, 1 mM PMSF, 5 μg/ml leupeptidin, 5 μg/ml pepstatin, 5 μg/ml aprotinin, 1mM Na_3VO_4, and 1 mM DTT. After centrifugation, tissue extracts were resolved by SDS-PAGE and were analyzed by immunoblot. The membranes were probed with Abs to phospho-JNK, phospho-p38MAPK, phospho-ERK, cleaved caspase-3, and β-actin as loading control (Cell Signaling Technology). Blots were developed with ECL plus (Amersham Biosciences).

Apoptosis

Fresh lung alveolar cells were pooled from five animals (5×10^5 fresh cells) for each group and were analyzed. Fresh whole blood was drawn from the inferior vena and pooled from five animals (1×10^6 fresh cells) for each group. After blocking with Fc block (BD Biosciences), murine IgG, and rat IgG (Jackson ImmunoResearch Laboratories), cells were stained with mAbs including the following: F4/80-allophycocyanin (Serotec), Gr1-FITC (BD Biosciences), 7-aminoactinomycin D, and annexin V-PE (BD Biosciences). Analysis was performed using FACSVantage SE (BD Biosciences), and counts were calculated automatically by FlowJo software (Tree Star). For the

TUNEL assay (DeadEnd Colorimetric TUNEL System, Promega), paraffin-embedded sections were deparaffinized by immersing slides in xylene and then through graded ethanol washes (100, 95, 85, 70, 60, and 50%), followed by a wash in 1× PBS. The tissue sections were then fixed by immersing the slides in 4% paraformaldehyde for 15 min. A proteinase K solution of 20 μg/ml was placed on the slides for 22 min to help permeabilize the tissue. The slides were then washed with 1× PBS and refixed in 4% paraformaldehyde for another 5 min and washed again. In situ nick end labeling of nuclear DNA fragmentation was performed in a humid chamber for 1 h in the dark at 37°C. A positive control slide was prepared by treating the tissue sections with RNase-free DNase for 10 min before the above labeling. A negative control slide was prepared by omitting the rTdT from the above labeling step. The labeling reaction was stopped by immersing the slides in 2× SSC for 15 min, followed by a 1× PBS wash. Endogenous peroxidases were blocked by immersing the slides in 0.3% hydrogen peroxide in PBS for 5 min and then washing with 1× PBS. Color was developed by diaminobenzidine. The slides were then mounted with Permount mounting medium (Fisher Scientific) and observed under a light microscope.

TLR4 immunohistology and flow cytometry

Whole lung lavage was performed to obtain three pools of two animals 24 h after exposure to ozone or FA. After blocking for 10 min on ice with Fc block, murine IgG, and rat IgG (Jackson ImmunoResearch Laboratories), live lavage cells were stained for surface expression of F4/80 (Serotec) and TLR4 for 30 min and washed. Alveolar macrophages were identified as F4/80$^+$. Surface expression of TLR4 was determined using PE-conjugated mAbs to TLR4 (clone U141, eBioscience). Cells were fixed with 1% ultrapure paraformaldehyde (Polysciences); flow cytometry analysis was performed using BD Biosciences LSR II flow cytometer; and data were analyzed with BD FACSDiva software (BD Biosciences). A laser-scanning confocal microscope (LSM 510 UV mounted on Axiovert 200M microscope; Zeiss) was used to obtain the fluorescence and differential interference contrast images. The images were obtained simultaneously using the 488- and 543-nm lasers as the light source, and the Zeiss Plan-Apo ×63 oil N.A. = 1.4 as objective lens. For fluorescence, either a 505- to 550-nm or a long pass 560-nm filter was used for the emission, with a pinhole of 1 Airy unit, corresponding to a z-resolution of 0.8 μm. The software used for acquisition was Zeiss LSM510 version 3, and for analysis, LSM Image Examiner version 3.2.

Statistics

Data are expressed as mean ± SEM. Significant differences between groups were identified by ANOVA. Individual comparisons between groups were confirmed by Student's *t* test, unless otherwise stated. Statistical calculations were performed using SPSS. A two-tailed *p* value of <0.05 was considered statistically significant.

Results

Ozone enhances airway hyperresponsiveness in response to inhaled LPS

To determine whether ozone could modify the innate immune response to LPS, we primarily exposed mice to either filtered air or inhaled ozone for 3 h (2 ppm), followed by secondary exposure to either saline or inhaled LPS at 24 h, 48 h, 72 h, or 7 days. All experiments follow the same experimental protocol for coexposures. Previously, both ozone and LPS inhalation have independently been shown to cause airway hyperresponsiveness (AHR) to inhaled methacholine. Ozone exposure alone caused enhanced AHR at early time points (24 and 48 h), and this response peaked at 24 h and returned to baseline by 72 h. However, 48 and 72 h after ozone exposure, the airway response to inhaled LPS was synergistically enhanced over the AHR

observed following either exposure alone (Fig. 1*C*). By 7 days after ozone exposure, the physiologic response to LPS was independent of ozone pre-exposure (data not presented). This observation suggests that the effects of ozone-priming response to LPS have, in part, resolved by 7 days after exposure. Therefore, ozone can enhance LPS-induced airway hyperreactivity to methacholine up to 72 h after exposure.

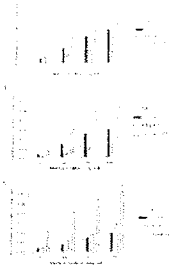

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FIGURE 1.

Ozone enhances airway hyperresponsiveness in response to LPS. Airway response to methacholine was measured at 24 (*A*), 48 (*B*), and 72 h (*C*) after pre-exposure. Ozone significantly increases airway hyperresponsiveness at 24 and 48 h (†, $p < 0.05$, FA vs ozone). LPS can enhance AHR (#, $p < 0.05$, FA vs LPS). Ozone modifies the response to LPS, which is evident by 48 h after ozone exposure, when we begin to notice enhanced response to inhaled LPS. Seventy-two hours after exposure to ozone, when the effects of ozone alone have returned to baseline, significant persistent enhanced response to inhaled LPS is present. (*, $p < 0.05$, FA-LPS vs ozone-LPS)

Ozone attenuates cellular inflammation in response to inhaled LPS

To determine whether ozone priming of the LPS response in the airways was dependent on cellular inflammation, we characterized recruitment of cells into the alveolar compartment after exposure. Unexpectedly, we observed significantly reduced LPS-induced pulmonary inflammation at both 24 and 48 h after pre-exposure to ozone (Fig. 2*A*). We have previously demonstrated that mice develop airways hyperresponsiveness after inhalation of ozone (22). Because it was conceivable that enhanced ozone-induced bronchoconstriction could minimize the effects of inhaled LPS by reducing the deposition and distribution of the aerosol, we challenged animals pre-exposed to FA or ozone to doses of LPS similar to aerosol by oropharyngeal aspiration (1.7 μg/50 μl saline). LPS delivered to the airways in this manner is largely independent of ozone-induced changes in air flow. The differences we observed in response between the groups using this method were identical with those using aerosolized LPS. Importantly, reduced concentrations of total cells (ozone-LPS, 6.1×10^4 vs FA-LPS, 16.5×10^4), macrophages (ozone-LPS, 1.0×10^4 vs FA-LPS, 1.7×10^4), and neutrophils (ozone-LPS, 5.0×10^4 vs FA-LPS, 14.7×10^4) persisted with this method of LPS administration. Therefore, we conclude that ozone pre-exposure can attenuate cellular inflammation in the lungs in response to aerosolized LPS independent of changes in airway hyperresponsiveness.

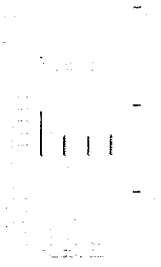

FIGURE 2.

Ozone pre-exposure reduces the number of inflammatory cells in the lung after exposure to LPS. Cell counts from bronchial alveolar lavage were determined at multiple time points after pre-exposure to either FA or ozone (O_3). We

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report total cell counts (*A*), absolute alveolar macrophages (*B*), and neutrophils (*C*). Ozone exposure alone induced significant increases in total cells, macrophages, and neutrophils at 24 h. FA-LPS-exposed animals demonstrate significant increases in total cells, macrophages, and neutrophils at all time points when compared with FA. Some animals were coexposed to inhaled LPS at various time points after pre-exposure to either FA or ozone. Ozone pre-exposure significantly reduced the numbers of total cells, macrophages, and neutrophils in the lungs of LPS-exposed animals at both 24 and 48 h after pre-exposure (*a*, $p < 0.05$, FA-LPS vs ozone-LPS). This represents one of three experiments ($n = 10$ per group).

Ozone enhances production of inflammatory markers in response to inhaled LPS

Although reductions in inflammatory cells in the lung caused by sequential exposure to ozone and LPS could have been explained by reduced production/release of cytokines and chemokines in the lower airway, this was not observed. In contrast, we found dramatic increases in the concentration of several proinflammatory proteins after pre-exposure to ozone, including the following: KC, MIP-1α, and IL-6 (Fig. 3⇓). Minimal, but significant increases in IL-10, IL-12(p70), and IL-17 were also seen with ozone pre-exposure at 24, 48, and 72 h (data not shown). TNF-α was significantly increased after ozone pre-exposure only at 72 h (1811 ± 459 vs 3614 pg/ml ± 521, $p < 0.05$). To specifically address whether any undetected secreted factors contribute to altered cellular migration, we harvested zymosan-elicited peritoneal neutrophils from naive animals. No significant differences were observed in the chemotactic response to lavage fluid obtained from ozone vs FA pre-exposed mice to explain an absolute reduction in recruitment of inflammatory cells (chemotaxis index, FA-LPS, 5.8 vs ozone-LPS, 5.7). This further suggests that alteration in secreted factors in the alveolus did not significantly contribute to reduced cell populations in the lung associated with ozone pre-exposure.

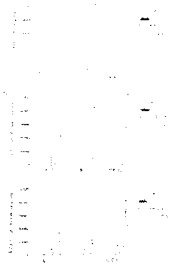

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FIGURE 3.

Ozone pre-exposure enhances cytokine production in the lung lavage after exposure to LPS. Cytokines were measured in the bronchial alveolar lavage fluid at 24 (*A*), 48 (*B*), and 72 h (*C*) after pre-exposure. Ozone alone caused significant increase in both IL-6 and KC at 24 h, when compared with FA ($p < 0.001$). After ozone exposure, KC remained significantly elevated at both 72 h and 7 days ($p < 0.01$). FA-LPS increased IL-6, KC, and MIP-1α at each time point tested ($p < 0.01$). Pre-exposure to ozone enhanced cytokine production in response to inhaled LPS, including the following: 24, 48, and 72 h (*a*, $p < 0.05$, FA-LPS vs ozone-LPS).

Similarly, we found that markers of LPS-induced lung injury were substantially increased in mice that had been previously exposed to ozone. Enhanced LPS-induced lung injury in ozone-exposed mice was supported by dramatic increases in the concentration of total protein in the lavage fluid (Fig. 4⇓*A*), a

higher percentage of circulating neutrophils (FA, 2.2%; ozone, 1.1%; FA-LPS 10.8%; ozone-LPS, 20.2%), and higher serum concentrations of IL-6 (Fig. 4*B*). Disruption of epithelial integrity and enhanced systemic inflammation could be associated with increased translocation of inhaled LPS into the systemic circulation. However, we were unable to detect significant differences in the concentration of LPS in the serum of coexposed animals (24 -h values: FA-LPS, 0.73 EU/ml vs ozone-LPS, 0.96 EU/ml; $p = 0.31$).

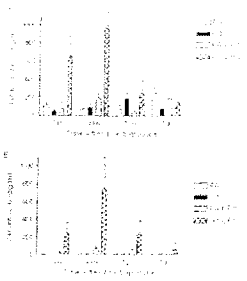

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FIGURE 4.

Ozone pre-exposure enhances total protein in the lung lavage and systemic IL-6 after exposure to LPS. Bronchial alveolar lavage fluid was measured for total protein at each time point (*A*). We did not observe significant increases in total protein in the lavage fluid after FA, ozone, or FA-LPS inhalation. Pre-exposure to ozone dramatically increased the level of total protein in the lavage when compared with FA-LPS at 24, 48, and 72 h (*, $p < 0.05$, FA-LPS vs ozone-LPS). Systemic inflammatory response was determined by measurement of serum IL-6 (*B*). LPS increased systemic IL-6 when compared with either FA or ozone; however, this was dramatically increased in the ozone-LPS group (*, $p < 0.05$, FA-LPS vs ozone-LPS).

Inhaled ozone reduces neutrophil spontaneous cytokinesis and induces apoptosis of macrophages in the lung and circulating blood monocytes

Reduced LPS-induced inflammatory cell recruitment into the lungs associated with ozone pre-exposure could be the result of either impaired cellular motility or enhanced cell death. To determine whether ozone directly impaired cellular migration, we harvested lung neutrophils from animals exposed to inhaled LPS with or without ozone pre-exposure. Cells were then analyzed for their migratory potential using Transwells (31). In the absence of any chemotactic signal, there was a 7.2-fold decrease in nonspecific migration (chemokinesis) in ozone-treated cells compared with those exposed to FA (data not shown). When a chemotactic gradient was added (LTB_4), similar increases in PMN migration from each of these groups were observed (chemotaxis index: FA-LPS, 1.5 vs ozone-LPS, 2.8). However, the absolute number of ozone-treated cells that migrated toward LTB_4 was reduced. Thus, a reduction in spontaneous neutrophil cytokinesis after ozone exposure resulted in an absolute reduction in the number of PMNs that migrated to LTB_4. This observation suggested that mice pre-exposed to ozone had a population of PMNs that were functionally unable to migrate to chemotactic stimulus.

To determine why subset of neutrophils demonstrated reduced spontaneous cytokinesis and to better understand the reduced numbers of macrophages recruited into the lung associated with pre-exposure to ozone, we explored the possibility that ozone exposure was inducing apoptosis or necrosis of inflammatory cells. To address this question, we performed flow analysis to discriminate populations of inflammatory cells in the lower airway and circulating blood 24 h after pre-exposure to ozone that might be undergoing cell death. Neutrophils recruited into the lung after inhaled LPS demonstrated a 40% increase in apoptosis when pre-exposed to inhaled ozone (ozone 4.2%; LPS, 3.6%; ozone-LPS, 6.0%). Interestingly, ozone exposure by itself had a profound effect on macrophages. We found that ozone alone can induce apoptosis in alveolar macrophages (FA, 7.8% vs ozone, 27%),

whereas inhaled LPS alone did not have a measurable impact on macrophage apoptosis at this time point (FA, 7.8% vs FA-LPS, 8.0%) (Fig. 5*A*). Mice were phenotyped at an early time point after exposure to LPS, which most likely explains the lack of LPS-induced apoptosis observed. However, LPS exposure in the context of ozone pre-exposure substantially increased the percentage of macrophages in the lung undergoing apoptosis (ozone, 27% vs ozone-LPS, 51%). Ozone-induced apoptosis of airway inflammatory cells in the lung was confirmed by TUNEL staining (Fig. 6) and an increase in cleaved caspase 3 (Figs. 7*E* and 8*E*). Secondary necrosis was observed after either ozone or LPS exposure (FA, 29.9%; ozone, 40.8%; FA-LPS, 35.6%; ozone-LPS, 26%). To determine whether the effects of ozone were limited to the lung, we examined apoptosis in leukocytes isolated from whole blood from animals in each of these groups. No significant difference in systemic neutrophil apoptosis was detected. However, ozone pre-exposure increased the concentration of apoptotic monocytes in the systemic circulation after exposure to LPS (FA, 3.8%; ozone, 4.6%; FA-LPS, 2.8%; ozone-LPS, 22.8%) (Fig. 5*B*). These data demonstrate that ozone pre-exposure not only enhanced apoptosis in leukocytes recruited to the lung in response to inhaled LPS, but also circulating monocytes, which might be involved systemically in enhancing and sustaining the initial inflammatory response.

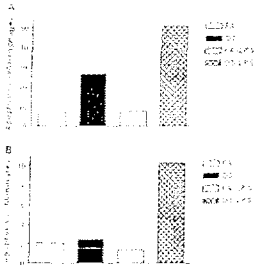

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FIGURE 5.

Ozone enhanced apoptosis of macrophages in the lung and blood. Live bronchial alveolar lavage cells were pooled from five animals in each group 24 h after pre-exposures. Flow cytometric analysis was performed and gated on alveolar macrophages (F4/80 positive). Pools were evaluated for necrosis (7-aminoactinomycin D positive) vs apoptosis (annexin V positive). Ozone alone increases the percentage of apoptotic alveolar macrophages (*A*). No increase in apoptosis was observed with LPS alone. Ozone pre-exposure dramatically increased the percentage of apoptotic macrophages after LPS coexposure in both alveolar macrophages (*A*). Whole blood was pooled from five animals in each group 24 h after pre-exposure. Circulating monocytes (F4/80 positive) from whole blood demonstrate enhanced apoptosis only after coexposure (*B*).

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FIGURE 6.

LPS caused robust neutrophil recruitment and ozone-enhanced apoptosis in macrophages. Histology was evaluated at 24 h after pre-exposures, including the following: FA (*A* and *B*), ozone (*C* and *D*), FA-LPS (*E* and *F*), and ozone-LPS (*G* and *H*). We present representative H&E staining (*A*, *C*, *E*, and *G*) and TUNEL staining (*B*, *D*, *F*, and *H*) at ×400 power. Moderate perivascular and subepithelial neutrophils are observed with exposure to LPS. Alveolar epithelial septal thickening is appreciated with exposure to ozone. Airway macrophages stain positive for TUNEL in both ozone and ozone-LPS groups (+, arrows).

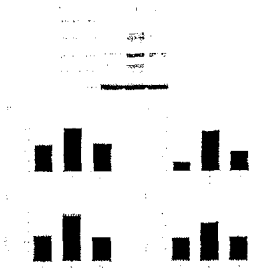

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FIGURE 7.

Ozone activates MAPKs immediately after exposure. Western blot analysis on lung homogenates was performed on animals before ozone exposure, 4 h after initiation of exposure, and 24 h after exposure (*A*). Densities of bands are quantified as normalized to B-actin in phospho-JNK (*B*), phospho-p38 (*C*), phospho-ERK (*D*), and cleaved caspase 3 (*E*). Statistically significant differences are identified (*, $p < 0.05$; two-tailed Mann-Whitney *U* test). Densitometry data represent mean ± SEM from three repeats.

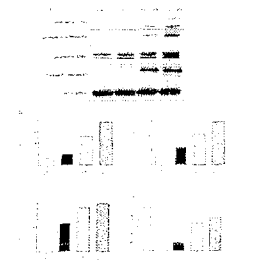

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FIGURE 8.

Ozone pre-exposure enhances LPS-dependent downstream signaling. Western blot analysis on lung homogenates was performed to determine the level of phosphorylation of proteins related to LPS-dependent signaling and apoptosis at 24 h after pre-exposure to either FA or ozone (*A*). For each gel, ozone pre-exposure increased the level of phospho-JNK1, phospho-p38, phospho-ERK, and cleaved caspase 3 when compared with LPS alone. Densitometry of the bands revealed the following. FA + LPS and O_3 + LPS were statistically significantly higher, when compared with either FA or O_3 for phospho-JNK1 (*B*), phospho-p38 (*C*), phospho ERK (*D*), and cleaved caspase 3 (*E*) (comparisons not specifically identified, $p < 0.05$; two-tailed Mann-Whitney *U* test). Significant differences between FA + LPS vs O_3 + LPS are labeled (*, $p < 0.05$; two-tailed Mann-Whitney *U* test). Densitometry data represent mean ± SEM from three repeats.

Ozone enhances LPS signaling in the lung

To determine whether ozone initiates intracellular signaling through innate immune pathways, we investigated the individual and combined effects of ozone and LPS on downstream signaling in whole lung homogenates. Ozone alone transiently increased total IL-1R-associated kinase-M (data not shown), as well as phosphorylation of JNK, ERK, and p38 4 h after exposure (Fig. 8*A*). This increase was statistically significant for p38. However, the enhanced signal associated with acute ozone exposure returned to baseline by 24 h after exposure. All proteins were significantly higher in mice exposed to either FA + LPS or O_3 + LPS when compared with mice exposed to either FA or O_3 ($p < 0.05$, Mann-Whitney *U* test). Mice pre-exposed to ozone showed enhanced phospho-JNK, phospho-ERK, and phospho-p38 immunoreactivity when compared with either exposure alone at the 24-h time point (Fig. 7*A*). We did not observe ozone-dependent differences in TLR4 protein expression

or in mRNA expression of known negative regulators of TLR4 signaling, including IRAK-M, A20, or tollip at this time point (data not shown).

Ozone exposure modifies the cellular distribution of TLR4

We did not observe differences in either mRNA or protein expression of TLR4 at 24 h after exposure to ozone that would explain ozone-induced enhanced TLR4 signaling (data not shown). For that reason, we considered that the spatial distribution of this receptor might account for the enhanced TLR4 signaling. In fact, 24 h after exposure to ozone, we observe increased intensity and altered distribution of TLR4 on alveolar macrophages by fluorescent microscopy (data not shown). Alveolar macrophages were identified by confocal microscopy ($F4/80^+$) and demonstrate robust enhanced expression of TLR4 after exposure to ozone (Fig. 9, *A–F*). This was quantified by a shift in the percentage of alveolar macrophages that express a high concentration of TLR4 as detected by flow cytometry and a shift in the median fluorescent intensity (Fig. 9, *G* and *H*). Moreover, confocal microscopy images indicate that inhaled ozone results in altered special distribution of TLR4 on alveolar macrophages. These observations suggest that enhanced TLR4 signaling is related to trafficking of TLR4 within alveolar macrophages in response to inhaled ozone.

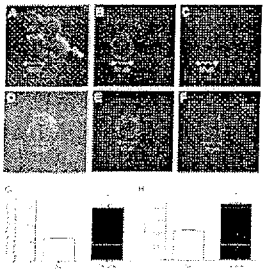

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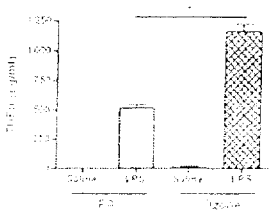
FIGURE 9.

Ozone modifies expression and cellular distribution of TLR4 on macrophages. Bronchial alveolar cells were analyzed by confocal microscopy. We identified alveolar macrophages by light microscopy (*A* and *D*) and the presence of the alveolar macrophage marker F4/80 (*B* and *E*). We demonstrate enhanced surface expression of TLR4 on alveolar macrophages after ozone exposure (*F*), when compared with FA controls (*C*). Three pools of two animals of live bronchial alveolar lavage cells were obtained 24 h after pre-exposure to either FA or ozone. Flow cytometric analysis was performed and gated on alveolar macrophages (F4/80 positive). Surface expression of TLR4 was determined by staining. Ozone increased the percentage of macrophage with increased surface expression of TLR4 (*G*) and increased the median fluorescent intensity (*H*).

Ozone enhances the biologic response by alveolar macrophages

Ozone enhanced LPS-induced production of proinflammatory cytokines in the whole lung, increased apoptosis of alveolar macrophages, and was associated with redistribution of TLR4 on alveolar macrophages. To specifically determine whether exposure to ozone modified the functional response of alveolar macrophages to LPS, we isolated lung macrophages from ozone-exposed animals by density centrifugation. These cells were subsequently challenged to LPS in vitro, and levels of TNF-α from the supernatant were measured. Pre-exposure to ozone does prime the innate immune response to endotoxin under these conditions (Fig. 10*A*). This observation further supports the association between ozone-induced altered distribution of cellular TLR4 and enhanced biological response to LPS.

FIGURE 10.



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Ozone enhances the response of lung macrophages to endotoxin. Pulmonary macrophages were isolated from mice exposed to either filtered air or ozone. Macrophages were exposed to either saline or endotoxin ex vivo for 4 h, and levels of TNF-α were measured in the supernatant. We demonstrate significant enhanced response to endotoxin in cells pre-exposed to ambient ozone (*, $p < 0.001$; FA-LPS vs O_3-LPS).

Discussion

Our findings indicate that inhalation of ozone substantially enhances innate immune responsiveness. Prior exposure to ozone results in greater LPS-induced airway hyperreactivity, higher concentrations of total protein, higher concentrations of proinflammatory cytokines, and an enhanced systemic inflammatory response to inhaled LPS. Ozone inhalation was associated with an enhanced biologic response to LPS, which was associated with enhanced proinflammatory signal and enhanced programmed cell death. Our results suggest that the enhanced biologic response to LPS is caused by altered cellular distribution of TLR4 following inhalation of ozone. In aggregate, these observations indicate that inhalation of ozone increases local and systemic LPS-induced injury by priming the innate immune system through altered special distribution of TLR4 and enhanced signaling.

It has long been appreciated that strict control of the biologic response to bacterial toxins is critical to host survival. This is highlighted by the fact that TLR4-deficient animals are protected against LPS-induced shock (32), yet are vulnerable to overwhelming five Gram-negative infections (33). Although it is essential to recognize, respond to, and clear pathogens, enhanced (acute or persistent) response to microorganisms can also lead to detrimental effects. In this study, we have demonstrated that ozone can substantially affect the response to LPS by priming the innate immune system through TLR4 cell surface expression. These findings are entirely consistent with the observed effect of ozone on decreased clearance of live bacterial pathogens (12, 17, 18, 19, 20, 21). Moreover, these findings provide a biological hypothesis for the epidemiological relationship between ozone and increased pulmonary morbidity and mortality. Loss of lung function in chronic obstructive pulmonary disease is related to exposure to environmental air pollutants (34, 35) and frequency of exacerbations (36). Our observations support that exposure to ozone can significantly enhance both lung injury and macrophage apoptosis after exposure to inhaled endotoxin. These findings suggest that ozone priming of response to inhaled endotoxin could contribute to both loss of lung function and defective antibacterial host defense.

Our results demonstrate that inhaled ozone can alter the spatial distribution of TLR4 and results in an enhanced response to LPS. This observation is consistent with previous in vitro observations, which demonstrate after stimulation trafficking of TLRs to the surface membrane of HEK293 cells (37). Recent observations in a model of hemorrhagic shock demonstrate that H_2O_2 can induce surface expression of TLR4 on monocytic cells (38). Furthermore, previous studies using transgenic animals demonstrate that the level of TLR4 RNA expression correlates with biologic function, suggesting a dose effect dependent on TLR4 expression (39). Our in vivo observations clearly demonstrate the pathophysiologic importance of exposure to inhaled environmental air pollutants and support that exposure to ambient ozone may have a profound effect on innate immune responsiveness. However, it

remains possible that other members of the receptor complex or downstream adaptor molecules could also be altered by inhaled ozone. For example, an increase in soluble CD14 after exposure to ozone has been observed in an experimental study in humans (40). CD14, a critical component of the TLR4 complex, can either enhance the biologic response to LPS or act as a sink attenuating this response. Improved understanding of the environmental factors that can regulate TLR4-dependent signaling will improve our understanding of host defense.

Precise regulation of TLR-dependent signal is required to optimize normal inflammation and resolution of injury. Although inhaled ozone activates many proinflammatory pathways, we found that it also accelerates apoptosis. Thus, enhanced apoptosis may serve to regulate uncontrolled innate immune response. It remains plausible that this is a protective mechanism, in that robust monocyte and neutrophil recruitment, in the absence of apoptosis, could magnify the severity of lung damage. Attenuated inflammatory cell recruitment after a severe oxidative lung injury could prove protective. We speculate that in the absence of macrophage apoptosis, there might have been even higher levels of proinflammatory cytokines, neutrophil recruitment, and lung injury. In fact, ozone-induced apoptosis could also explain how pre-exposure to ozone can be protective with influenza infection (41), a disease in which acute morbidity is associated with an uncontrolled inflammatory response. In contrast, recent work supports that low-level TLR4 signaling appears protective in other forms of oxidative lung injury (42, 43, 44). Combined, these observations support divergent mechanisms of strict regulation of innate immune response to limit oxidative lung injury dependent on the level of activation.

It is noteworthy to highlight that the physiologic response of the airways was independent of the concentration of inflammatory cells. However, secreted cytokines/chemokines were clearly up-regulated with coexposure, as are other markers of lung injury. Many factors, including the cytokines IL-1β (45), TNF-α (46, 47, 48), and IL-6 (49), have been associated with ozone-related AHR. In our model of coexposure, IL-6 was dramatically increased in both the lavage fluid and serum at all time points tested. Although it remains unclear whether this specific cytokine accounts for the differences in TLR4-dependent AHR, this study further supports the dichotomy between cellular inflammation and AHR. Further studies are needed to identify the pathogenesis of airway hyperresponsiveness, which remains a hallmark of asthma.

Ozone is a common urban air pollutant that significantly contributes to increased pulmonary morbidity and mortality, especially among those with chronic lung disease (1, 2, 3, 4). We have demonstrated that ozone can prime the biologic response to inhaled LPS, leading to enhanced airway injury and apoptosis of inflammatory cells in the lung. TLR4-dependent signaling in the lung appears to be a double-edged sword. A controlled response is critical for effective clearance of bacterial pathogens. However, an exaggerated response can be associated with an increase in airway hyperresponsiveness, airway injury, and reduced numbers of functional inflammatory cells. If we are to gain a better understanding of disease etiology and pathogenesis, we need a more detailed understanding of the complex interaction between common environmental exposures and fundamental homeostatic mechanisms that regulate innate immunity.

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Disclosures

The authors have no financial conflict of interest

Footnotes

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..i2 Address correspondence and reprint requests to Dr. John W. Hollingsworth, Division of Pulmonary, Allergy, and Critical Care Medicine, Duke University Medical Center, Box 3136, Durham, NC 27710. E-mail address: holli017@mc.duke.edu

..i3 Abbreviations used in this paper: COPD, chronic obstructive pulmonary disease; AHR, airway hyperresponsiveness; FA, filtered air; LTB_4, leukotriene B_4; PMN, polymorphonuclear cell; ppm, parts per million.

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β-arrestin Deficiency Protects Against Pulmonary Fibrosis in Mice and Prevents Fibroblast Invasion of Extracellular Matrix

Alysia Kern Lovgren[1], Jeffrey J. Kovacs[2], Ting Xie[1,3], Erin N. Potts[1], Yuejuan Li[1], W. Michael Foster[1], Jiurong Liang[1], Eric B. Meltzer[1], Dianhua Jiang[1], Robert J. Lefkowitz[2,4,5], and Paul W. Noble[1,*]

[1] Division of Pulmonary, Allergy, and Critical Care Medicine, Departments of Medicine, Duke University Medical Center, Durham, NC 27710, USA

[2] Department of Medicine, Duke University Medical Center, Durham, NC 27710, USA

[3] Beijing University of Chinese Medicine, Beijing, China

[4] Department of Biochemistry, Duke University Medical Center, Durham, NC 27710, USA

[5] Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710, USA

Abstract

Idiopathic pulmonary fibrosis (IPF) is a progressive disease causing unremitting extracellular matrix deposition with resultant distortion of pulmonary architecture and impaired gas exchange. β-arrestins regulate G-protein-coupled receptors through receptor desensitization while acting as signaling scaffolds that facilitate numerous effector pathways. Here we examine the role of β-arrestin1 and β-arrestin2 in the pathobiology of pulmonary fibrosis. In the bleomycin-induced mouse lung fibrosis model, loss of either β-arrestin1 or β-arrestin2 results in protection from mortality, inhibition of matrix deposition, and protected lung function. Fibrosis is prevented despite preserved recruitment of inflammatory cells and fibroblast chemotaxis. However, isolated lung fibroblasts from bleomycin-treated β-arrestin null mice fail to invade extracellular matrix while displaying altered expression of genes involved in matrix production and degradation. Furthermore, knockdown of β-arrestin2 in fibroblasts from IPF patients attenuated the invasive phenotype. These data implicate β-arrestins as mediators of fibroblast invasion and development of pulmonary fibrosis, thus representing a potential target for therapeutic intervention for patients with IPF.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive lung disease of unknown etiology that leads to loss of lung function (1, 2). IPF is characterized by patchy subpleural parenchymal fibrosis with pathological features including accumulation of myofibroblasts,

*To whom correspondence should be addressed. paul.noble@duke.edu

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Author contributions: A.K.L. conceived, designed, performed, and analyzed most experiments and wrote the manuscript. J.J.K. conceived and performed the gene expression experiments, analyzed the data, and wrote the manuscript. E.N.P. performed the lung mechanics experiments. J.X. performed the knockdown experiments and analyzed the data. Y.L. developed the invasion assay protocol that was used. J.L. helped with the invasion experiments. E.B.M. purified human lung fibroblasts from IPF patients. W.M.F. in whose laboratory the lung mechanics experiments were performed provided funding for those experiments. D.J. conceived experiments, analyzed the data, and wrote the manuscript. R.J.L. in whose laboratory the gene expression experiments were conceived and performed, provided the knockout animals, provided funding for experiments, and wrote the manuscript. P.W.N. conceived and provided funding for all experiments and wrote the manuscript.

formation of fibroblast foci, distortion of pulmonary architecture, and increased collagen deposition (1, 3).

After injury to the airway epithelium, important profibrotic mediators, such as transforming growth factor-β (TGF-β) and matrix metalloproteinases (MMPs), are released by myofibroblasts and the epithelium. There is abundant evidence that TGF-β is critical for the progression of pulmonary fibrosis in mice due to its role in regulating collagen synthesis, fibroblast proliferation, apoptosis, and myofibroblast differentiation (4–6). MMPs have been implicated in extracellular matrix remodeling and basement membrane disruption, which allows fibroblasts to invade into the alveolar space where they proliferate, form foci, and produce collagen (3, 7, 8). When dysregulated, a decrease in fibroblast/myofibroblast apoptosis and increase in fibroblast/myofibroblast activity leads to thickening and stiffening of the septal walls, which reduces the ability to transport oxygen into the capillaries and ultimately results in respiratory failure.

β-arrestins are ubiquitously expressed members of the arrestin protein family. Studies show that two of the arrestin isoforms, termed β-arrestin1 and β-arrestin2, have non-redundant roles; however, some functional redundancy has been observed depending on the situation. β-arrestins are classically known to regulate G protein-coupled receptor (GPCR) signaling through receptor desensitization and internalization. More recently, β-arrestins have been shown to be important signaling scaffolds that facilitate the activation of numerous effector pathways, such as the mitogen-activated protein kinases (MAPKs) and Akt (9). Appreciation of the number of GPCR signaling pathways that are engaged through the β-arrestins has grown rapidly, and recent publications have also documented roles for the β-arrestins in signaling and/or endocytosis of other cellular receptors such as the single membrane-spanning TGFβ receptors and the non-classical seven-transmembrane receptors Frizzled and Smoothened (10, 11). As with the GPCRs, many of these molecules are shown to interact with the β-arrestins in a ligand- or stimulus-dependent fashion (9, 11). With the emerging appreciation of β-arrestins as signaling mediators downstream of numerous classes of receptors, their demonstrated roles in mediating physiological responses have been expanding rapidly.

The mechanisms that regulate the progression of pulmonary fibrosis are not well understood. Currently, no medical therapies exist to increase life expectancy or improve quality of life, and median survival from the time of diagnosis is only two to three years (12). Prevailing hypotheses suggest that IPF is an epithelial-fibroblastic disorder that results from numerous microinjuries to the alveolar epithelia that lead to an impaired fibrotic repair response. Further research is necessary to enhance our understanding of the molecular mechanisms that play an important role in the aberrant fibroblast response and re-epithelialization after injury. Due to their vital role in numerous cell signaling mechanisms, β-arrestins have been implicated in a broad range of diseases including asthma and cancer (13, 14). The underlying mechanisms for progression of these diseases, such as cell recruitment and motility, may also be important in the development of fibrotic lung disease. However, the ability of β-arrestins to regulate fibroblast activity in pathological settings has not been investigated.

To elucidate the potential role of β-arrestins in the development of pulmonary fibrosis and fibroblast regulation, we used the well-established bleomycin mouse model of pulmonary fibrosis (15, 16). Traditional histological and biochemical techniques as well as physiological measurements of lung mechanics were used to demonstrate that both β-arrestin1 $^{-/-}$ and β-arrestin2 $^{-/-}$ mice are protected from the excessive collagen deposition, architectural distortion, and reduced lung compliance that result after bleomycin treatment. In addition, we investigated the inflammatory response and the behavior of primary lung

fibroblasts to better understand the mechanism involved in this protection. Our studies reveal an important role for β-arrestins in regulating fibroblast invasion and collagen formation in bleomycin-induced pulmonary fibrosis and suggest directions for development of novel therapeutic approaches.

RESULTS

β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ mice are protected from bleomycin-induced pulmonary fibrosis

To investigate the role of β-arrestin in the progression of pulmonary fibrosis, we used mice deficient in either β-arrestin1 or β-arrestin2 in the well-established bleomycin mouse model of lung fibrosis (15, 16). Intratracheal bleomycin administration (2.5 U/kg) results in approximately 50% mortality in wild-type (WT) mice within 21 days after treatment. However, as shown in Fig. 1A, both the β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ mice were remarkably protected from mortality. In congruence with the survival curve data, the WT bleomycin-treated mice had severe architectural changes as well as abundant collagen deposition upon histological examination. However, the lungs from the β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ mice demonstrated minimal collagen deposition and a histological appearance similar to untreated mice (Fig. 1B).

To further understand this protective mechanism, we used a lower dose of bleomycin (1.25 U/kg) that does not result in substantial mortality, which allowed us to study the entire cohort of mice at later time points. As previously described for this model, the WT mice had a significant increase in collagen deposition 21 days after bleomycin administration as quantitated biochemically by measuring hydroxyproline levels. Consistent with the lung histology results, the β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ mice had significantly less hydroxyproline in whole lung homogenates compared to the WT mice after bleomycin (Fig. 2A).

In humans, interstitial fibrosis leads to stiffening of the alveolar walls and a decrease in compliance (2). Similarly, bleomycin treatment has been shown to cause a significant decrease in static compliance in WT mice (17). Using a computer-controlled small animal ventilator, we generated pressure-volume curves and determined static compliance. As expected, WT mice had a significant decrease in static compliance values 21 days after bleomycin administration at 1.25 U/kg (Fig. 2B). Interestingly, the bleomycin-treated β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ mice had static compliance values similar to the saline control mice. Together with the histological and biochemical data, this suggests that the β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ mice are protected from the excessive collagen deposition, architectural changes, and stiffening of the lungs that normally occur after bleomycin treatment.

β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ mice exhibit normal recruitment of inflammatory cells after bleomycin administration

The bleomycin model of pulmonary fibrosis is characterized by an initial influx of inflammatory cells in response to injury. The magnitude of this initial inflammatory response is often correlated with enhanced fibrosis at later stages (18). To determine if loss of β-arrestins alters inflammatory cell recruitment, we collected bronchoalveolar lavage fluid (BALF) from WT, β-arrestin1$^{-/-}$, and β-arrestin2$^{-/-}$ mice 7 days after administration of 1.25 U/kg of bleomycin. Although the β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ mice are protected from the fibrotic response after bleomycin administration, they had a similar number of total inflammatory cells in BALF compared to WT mice (Fig. 3A). Additionally, histological analysis of lung sections from the bleomycin-treated WT and β-arrestin2$^{-/-}$

mice show similar patterns of inflammatory cells within the lung parenchyma (Fig. 3B). To determine whether the composition of the inflammatory influx differed between groups, differential staining was performed to identify the leukocyte populations. Again, no differences were observed between the WT, β-arrestin1$^{-/-}$, and β-arrestin2$^{-/-}$ mice in the ability to recruit macrophages, neutrophils, or lymphocytes (Fig. 3A). These data suggest that the inflammatory response after bleomycin treatment in β-arrestin$^{-/-}$ mice is preserved and that the protection from bleomycin-induced fibrosis in these animals may be due to another factor.

Loss of β-arrestin1 or β-arrestin2 does not alter TGF-β responsiveness in fibroblasts after bleomycin treatment

TGF-β plays a key role in the development of pulmonary fibrosis. In addition, β-arrestin2 has been shown to regulate TβRII internalization and subsequent TGF-β signaling (19). To determine if the protection of the β-arrestin$^{-/-}$ mice is due to loss of TGF-β responsiveness, we isolated primary lung fibroblasts from WT, β-arrestin1$^{-/-}$, andβ-arrestin2$^{-/-}$ mice 7 days after administration of 1.25 U/kg of bleomycin. Fibroblasts were treated with 5–10 ng/ml of TGF-β1 for 60 minutes, and total protein was isolated from the fibroblasts for Western blot analysis. As shown in Fig. 4A and 4B, the β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ fibroblasts had similar phospho-SMAD3 levels compared to the WT fibroblasts.

To further investigate TGF-β signaling after loss of β-arrestins, we next examined the production of a well-known TGF-β-stimulated matrix component, the glycosaminoglycan, hyaluronan (HA). Primary lung fibroblasts from WT, β-arrestin1$^{-/-}$, and β-arrestin2$^{-/-}$ mice were stimulated with 1–5 ng/ml of TGF-β1. After 24 hours, the media was removed and analyzed for total HA content by ELISA. Similar to the Western blot analysis of phospho-SMAD3, the fibroblasts lacking either β-arrestin1 or β-arrestin2 had an increase in HA production after TGF-β stimulation equivalent to the increase in the WT fibroblasts suggesting that the ability of fibroblasts to respond to TGF-β is not dependent on β-arrestin1 or β-arrestin2 (Fig. 4C). Additionally, we examined other downstream potentiators of TGF-β signaling that are important for the development and maintenance of pulmonary fibrosis such as PAI-1 and collagen 1. Fibroblasts were isolated from bleomycin-treated mice and then treated with 5 ng/ml of TGF-β 1 (Fig. 4D). Both PAI-1 and collagen 1 levels increased after TGF-β1 treatment in WT, β –arrestin1$^{-/-}$and β –arrestin2$^{-/-}$ cells. These data confirm the preserved signaling downstream of TGF-β pathways in the bleomycin-induced mouse model of IPF and suggest that the prevention of pulmonary fibrosis in the absence of β-arrestin is not due to impaired responsiveness to TGF-β.

Primary lung fibroblasts from β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ mice migrate normally in response to BALF

Since fibroblasts lacking β-arrestin1 or β-arrestin2 appeared to have normal TGF-β-induced signaling responses, we next hypothesized that these fibroblasts may be impaired in their ability to migrate to the site of injury. Lysophosphatidic acid, a component of BALF that increases after injury, mediates fibroblast migration by signaling through a specific G protein-coupled receptor, LPA1 (20). Loss of β-arrestins could potentially alter receptor internalization and thus cell surface expression of LPA1 or other receptors important in fibroblast chemotaxis. To investigate whether loss of β-arrestins could affect fibroblast migration we used modified Boyden chambers to measure the chemotaxis of primary lung fibroblasts towards BALF. As demonstrated in Fig. 5, the migratory ability of both the β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ fibroblasts in response to BALF from bleomycin-treated mice was similar to that of the WT fibroblasts. These data suggest that β-arrestins may not regulate fibroblast migration under these experimental conditions.

Primary lung fibroblasts from the β-arrestin1 $^{-/-}$ and β-arrestin2 $^{-/-}$ mice display a less invasive phenotype

The ability of fibroblasts to invade through the basement membrane and deposit excess collagen into the interstitium may be an important contributor to the progression of pulmonary fibrosis. White et al. demonstrated that, unlike normal lung fibroblasts, fibroblasts from patients with IPF spontaneously invade basement membranes (21). Consequently, we wondered whether fibroblasts that were isolated from bleomycin-treated mice would invade the basement membrane. Using the matrigel transwell assay, we found that fibroblasts from WT mice treated with bleomycin invaded the basement membrane matrix while fibroblasts from untreated mice had minimal invasive capacity. Since β-arrestin has been shown to play a role in cancer cell invasion (14), we hypothesized that the protective effect mediated by loss of β-arrestin function in mice subjected to the bleomycin-induced fibrosis model may be the result of a reduced invasive capacity of lung fibroblasts. To this end, we compared the invasive capacity of lung fibroblasts isolated from the WT, β-arrestin1$^{-/-}$, and β-arrestin2$^{-/-}$ mice after bleomycin treatment. Interestingly, as shown in Fig. 6A, the fibroblasts from the bleomycin-treated β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ mice were significantly less invasive than the WT fibroblasts, and their invasive capacity was more similar to the fibroblasts from the untreated control mice. Acute knockdown of β-arrestins from isolated WT fibroblasts through transient transduction of siRNAs specific for either β-arrestin1 or β-arrestin2 (Fig. 6B) recapitulated the loss of invasiveness seen in knockout mouse fibroblasts, suggesting that removal of either β-arrestin might serve as a therapeutic intervention for pulmonary fibrosis.

To determine if either β-arrestin1 or β-arrestin2 could contribute to the invasive phenotype of primary lung fibroblasts isolated from patients with IPF, we performed acute knockdown of β-arrestins. Knockdown of β-arrestin2 was more complete than β-arrestin1 in IPF fibroblasts (Fig. 7A) and resulted in an inhibition of fibroblast invasion in 5/5 patient samples (Fig. 7B). The effect of β-arrestin1 on IPF fibroblast invasion was less consistent and inhibition was observed in 2/4 IPF fibroblast samples resulting in a trend that did not achieve statistical significance. These data suggest that β-arrestins are important in mediating fibroblast invasion in both mouse and man although the relative contributions of β-arrestin1 and β-arrestin2 may not be identical between species. These data suggest that β-arrestins are necessary for fibroblast invasiveness during tissue injury, and the reduced invasive capacity of β-arrestin-deficient fibroblasts may indicate a possible target for therapeutic intervention.

Loss of β-arrestin1 or β-arrestin2 in primary lung fibroblasts results in altered expression of genes involved in matrix production, basement membrane degradation, and cell adhesion

To gain additional insights into the mechanisms by which loss of the β-arrestins causes reduced fibroblast invasion and protection from bleomycin-induced pulmonary fibrosis, we used quantitative RT-PCR to analyze gene expression in fibroblasts isolated from the bleomycin-treated WT, β-arrestin1$^{-/-}$, and β-arrestin2$^{-/-}$ mice. Our hypothesis was that the non-invasive β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ fibroblasts would have decreased expression of genes important in basement membrane degradation, such as the matrix metalloproteinases, or cell-matrix adhesion, such as the integrins. We analyzed 84 genes by using a targeted qRT-PCR array for extracellular matrix and adhesion proteins. In the β-arrestin1$^{-/-}$ fibroblasts, ten genes were significantly downregulated compared to WT fibroblasts. These genes included the following: *Emilin1*, *Col5a1*, *Lama1*, *Cdh1*, *Cdh4*, *Fbln1*, *Cnna2*, *Adamts2*, *MMP1a*, and *Thbs2* (Fig. 8). In the β-arrestin2$^{-/-}$ fibroblasts, a similar group of genes was downregulated, and the following five genes were significantly different from WT: *Col5a1*, *Lama1*, *Cdh1*, *Col1a3*, and *Spock* (Fig. 8). Only one gene,

Lama3, was significantly upregulated in the β-arrestin1$^{-/-}$ fibroblasts compared to the WT fibroblasts (Fig. 8). This analysis indicates that a number of genes involved in extracellular matrix production and remodeling are altered in their expression patterns in β-arrestin$^{-/-}$ mice after bleomycin treatment compared to WT mice. This change in gene expression may be related to one or more signaling pathways that are compromised by the loss of β-arrestins. The decrease in activity of these pathways likely contributes to the decrease in invasion by fibroblasts from β-arrestin$^{-/-}$ animals after bleomycin treatment.

DISCUSSION

IPF is a fatal disorder characterized by progressive fibrosis that leads to respiratory failure. The hallmark characteristics of IPF include fibroblast foci and increased collagen and extracellular matrix deposition. Although our understanding of the disease has increased over the last decade, an efficacious treatment remains elusive. Recent reviews have emphasized the importance of profibrotic mediators and alterations in fibroblast characteristics in disease pathogenesis (3). In this study, we demonstrate the importance of β-arrestins, ubiquitous mediators of cell signaling, in the development of bleomycin-induced pulmonary fibrosis. In addition, our data demonstrate a previously unidentified role for the β-arrestins in regulating certain aspects of fibroblast behavior.

Using the bleomycin mouse model of pulmonary fibrosis, we show that loss of function of either β-arrestin1 or β-arrestin2 protected mice from the excessive collagen deposition, architectural distortion, and decreased compliance that occur after bleomycin treatment. We show by physiological, biochemical, and pathological analysis that bleomycin-treated β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ mice were more similar to saline-treated mice than the bleomycin-treated WT mice. These findings confirm the necessity of β-arrestins for the development of bleomycin-induced pulmonary fibrosis. Furthermore, qRT-PCR analysis of primary lung fibroblasts from bleomycin-treated mice revealed decreased expression of genes involved in extracellular matrix production in the β-arrestin-deficient fibroblasts. This included genes that encode proteins important for collagen production such as collagen type IV α3, collagen type V α1, and a disintegrin- like and metallopeptidase with thrombospondin type1 motif 2 (Adamts2). Adamts2 is a procollagen N-proteinase that processes several types of procollagen proteins to mature collagen and is also important for collagen fibril assembly in the extracellular matrix. Interestingly, Adamts2-null mice are protected from carbon tetrachloride-induced hepatic fibrosis (22).

In addition to genes involved in collagen production, the primary lung fibroblasts from knockout animals treated with bleomycin also displayed decreased expression of genes that encode extracellular glycoproteins including thrombospondin 2, emilin, and laminin-α1 in comparison to WT mice. Mice deficient in thrombospondin 2 display abnormal collagen formation, and mice deficient in emilin have defective elastic fiber formation (23, 24). Together, these data suggest an important role for β-arrestins in modulating signaling pathways that regulate collagen and extracellular matrix production and formation mediated by fibroblasts after the onset of fibrosis.

Recently, a new hypothesis has emerged that links IPF and cancer due to their similar hallmark pathological alterations such as aberrant myofibroblast proliferation and apoptosis, extracellular matrix invasion, and intracellular signaling (25). One study has challenged current paradigms by demonstrating that the fibroblast foci of IPF form an interconnected, highly complex reticulum or neoplasm (26). Interestingly, the invasion of ovarian cancer cells was shown to be dependent on β-arrestin as a scaffolding protein necessary for endothelin-induced, β-catenin signaling (11). In the present study, we demonstrated that primary fibroblasts from bleomycin-treated β-arrestin-deficient mice had a less invasive

phenotype than their WT counterparts. Importantly, we were able to confirm a critical role for β-arrestins in fibroblast invasion by knocking down either β-arrestin1 or 2 in fibroblasts isolated from WT mice treated with bleomycin and thwarting the invasive phenotype. Furthermore, we provide data suggesting that β-arrestins may contribute to the invasiveness of fibroblasts isolated from human IPF patients, although more studies are needed in this area due to the heterogeneity of IPF fibroblasts.

Invasive cells must alter cell-cell and cell-extracellular matrix adhesions, degrade the extracellular matrix, and rearrange the actin cytoskeleton (27). Our qRT-PCR analysis revealed alterations in a number of genes that have been implicated in cancer invasion including MMP1a, Adamts2, Spock, Thbs2, Lama1, and Lama3. Matrix metalloprotease 1 (MMP1), a secreted enzyme that breaks down interstitial collagen, promotes the invasion of breast carcinoma and melanoma cells (28, 29). Increased expression of MMP1 has also been linked to the metastatic ability of colorectal cancer, and specific inhibition of MMP1 prevents metastasis of invasive melanomas (30). In addition, mice deficient in tissue inhibitors of metalloproteinase 3 (Timp3) have increased expression of MMP1a and enhanced pulmonary fibrosis after bleomycin treatment (31), suggesting that a decrease in MMP1a expression would have the opposite effect. Expression of Adamts2, another enzyme in the metalloprotease family, is upregulated in highly invasive mouse mammary tumors (32). Sparc/osteonectin, CWCV and kazal-like domain proteoglycan 1 (Spock1) is upregulated in prostate cancer and malignant thyroid tumors (33–35). Interestingly, Spock1 was also upregulated in mice that developed profibrotic-like changes in the lung after spaceflight (36). Thrombospondin 2 (Thbs2) is elevated in epithelial ovarian tumors and endometrial cancer samples from patients with a poor prognosis as well as at the invasive front of hepatocellular carcinomas (37–39). In addition, thrombospondin 2 has been shown to promote the invasiveness of pancreatic cancer cells (40). Interestingly, although both are basement membrane constituents, laminin-$\alpha 1$ (Lama1) has increased expression in glioblastomas and has been shown to promote invasion of melanoma cells while laminin-$\alpha 3$ (Lama3), the only upregulated gene in β-arrestin-deficient fibroblasts, is actually downregulated in both breast carcinoma and adenocarcinoma (41–44). Taken together, it seems that genes that have altered expression in β-arrestin-deficient fibroblasts are important for cellular invasion on multiple fronts and may indicate another link between IPF and metastatic cancers.

Our data suggest a previously unrecognized important role for β-arrestins in regulating fibroblast activity and collagen formation; however, we cannot rule out other potential mechanisms of β-arrestin-mediated signaling that contribute to the development of pulmonary fibrosis due to the complexity of β-arrestin signaling cascades. For example, loss of β-arrestin2 promoted re-epithelialization in a wound healing model, and the phenotype was attributed to increased CXCR2-mediated neutrophil recruitment and activity (45). In our model of bleomycin-induced injury, neutrophil recruitment, as well as macrophage and lymphocyte recruitment, was not altered in the β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ mice, so the mechanism appears to be different. Consistent with our finding,β-arrestin2$^{-/-}$ mice were shown to have a normal inflammatory cell response to LPS suggesting that these mice do not have a universal defect in inflammatory cell recruitment (13). Thus, our data do not support an impact on the inflammatory response as the protection incurred by loss of β-arrestins. In wound healing, other cell types besides neutrophils, such as keratinocytes and fibroblasts, also play vital roles. However, the ability of β-arrestins to regulate fibroblast activity during re-epithelialization was not investigated in the study by Su et al. (45). It is interesting to note that migration, and not invasion, of fibroblasts is important in wound healing, and our data demonstrate that there is no difference in the ability of fibroblasts lacking β-arrestins to migrate in response to BALF. In addition, the β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ fibroblasts responded normally to stimulation with TGF-β, a critical regulator of

fibroblast production of extracellular matrix during wound healing (46). Together, this suggests that fibroblasts lacking β-arrestins do not have an overall impairment in motility or cytokine responsiveness even though their ability to specifically invade basement membrane is impeded.

β-arrestins function as multiprotein scaffolds to coordinate complex signal transduction networks. One of the most studied of these β-arrestin-mediated signaling pathways is the ERK MAPK signaling axis. β-arrestin increases ERK activation through a G protein-independent pathway. However, β-arrestin-dependent ERK does not have typical nuclear functions, and specific downstream targets remain unknown although reports have suggested that cytosolic ERK may play a role in cytoskeletal rearrangement and chemotaxis (9). Interestingly, β-arrestin was shown to be a necessary scaffolding protein for activation of RAF-MEK-ERK1/2 signaling and MMP1 transcriptional activation in human bronchial epithelia, and this signaling pathway is also responsible for increased MMP1 expression in invasive melanoma cells (29, 47). In our study, the reduced expression of MMP1 as well as other genes in the fibroblasts lacking β-arrestins may also be due to a loss of β-arrestin-dependent ERK phosphorylation. However, due to the dramatic phenotype observed in the β-arrestin-deficient mice, we believe that more than one mechanism is responsible for this profound protection. As mentioned previously, β-arrestins may play a role in fibroblast invasion through activation of β-catenin signaling, as in ovarian cancer cells (14). In addition, the classical role of β-arrestins in receptor desensitization and internalization may also be important in the protection observed in the β-arrestin-deficient mice.

The data presented here demonstrate that both β-arrestin1 and β-arrestin2 play a pivotal role in the pathogenesis of bleomycin-induced pulmonary fibrosis. This suggests that both isoforms have a significant and non-redundant function in disease progression with possible mechanisms including the necessity for heterodimerization of both β-arrestins or the necessity of each in completely independent processes. There are numerous pathways that signal through β-arrestins (11), and consequently, genes that are dysregulated in fibroblasts isolated from bleomycin-treated mice could be downstream of any number of aberrant signaling pathways. Furthermore, at this early stage it can be hypothesized that β-arrestins are functioning in the same pathways redundantly, in some pathways synergistically, or actually opposing one another's function in the pathways examined, as all these signaling permutations have been observed (9). However, the gene expression profiles for these isolated fibroblasts appear distinct in that some genes are changed in only β-arrestin1$^{-/-}$ animals, some genes only in β-arrestin2$^{-/-}$ animals, and some genes are changed similarly in both knockouts. Consequently, it seems a reasonable hypothesis that there are signaling differences between these two genotypes, though these differences have yet to be fully elucidated.

IPF is a deadly disease that currently has no known effective therapy. Recent reviews have emphasized that IPF is not only an epithelial-fibroblastic disorder but also a disease with many similarities to cancer (3, 25). Thus, novel therapeutic agents that target fibroblasts and cancer-like mechanisms, such as invasion, warrant investigation. Our studies demonstrate that loss of β-arrestins protects against the development of pulmonary fibrosis by limiting the ability of fibroblasts to invade the basement membrane and deposit excess collagen and extracellular matrix. Furthermore, we demonstrate that acute removal of either β-arrestin from diseased mouse fibroblasts and β-arrestin2 in human IPF fibroblasts causes a significant reduction in the pathologic potential of these cells. Taken together, these findings suggest that locally delivered β-arrestin inhibitors may be a potential therapeutic for IPF as well as other causes of pulmonary fibrosis.

MATERIALS AND METHODS

Animals

β-arrestin1 $^{-/-}$ and β-arrestin2 $^{-/-}$ mice were generated as previously described (48, 49). All experiments were carried out using 8- to 12-week-old mice that had been backcrossed onto the C57BL/6 background for at least 12 generations. All studies were conducted in accordance with NIH guidelines for the care and use of animals and with approval from the Duke University Animal Care and Use Committee.

Bleomycin administration

Under anesthesia, either 1.25 U/kg or 2.5 U/kg bleomycin dissolved in sterile PBS or sterile PBS alone as a control was administered via oropharyngeal aspiration as previously described (50).

Histology

Twenty-one days following bleomycin treatment, the lungs were inflated with 10% formalin via a tracheal cannula and removed from the thoracic cavity. Tissue was fixed overnight, embedded in paraffin, and sectioned for staining with hematoxylin-eosin and trichrome.

Lung Mechanics

Static compliance measurements were generated as previously described (17). Briefly, mice were anesthetized, tracheostomized, paralyzed, and mechanically ventilated with a computer-controlled small-animal ventilator (Scireq, Montreal, Canada). Custom designed software (Flexivent, Scireq) was used to generate pressure-volume curves and calculate static compliance using the Salazar-Knowles equation.

Hydroxyproline

After assessment of lung mechanics, the whole lung was removed and total collagen content was measured with a conventional hydroxyproline method (51).

Bronchoalveolar lavage

Whole lung lavage was performed as previously described (52). The total number of cells in the BALF was determined using a hemacytometer, and differential cell counts were performed on cytospin preparations stained with Protocol Hema3 stain set.

Fibroblast Isolation

Mouse lung fibroblasts were isolated as previously described (53). The lungs from either unchallenged mice or mice 7 days after bleomycin treatment were minced, digested twice for 30 min at 37°C in digestion buffer (DMEM with 100 μg/ml of DNase I, 1 mg/ml Type IV collagenase, and 1 mg/ml BSA), passed through a 100 μm filter, centrifuged at 1500 rpm for 10 min., and plated in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Fibroblasts were cultured for ~10–12 days, and experiments were performed at passage 3–5.

Human lung fibroblasts were isolated from surgical lung biopsies or lung transplant explants obtained from patients with IPF (53). The diagnosis of IPF was arrived at by standard accepted American Thoracic Society recommendations (54). The specimens were obtained under the auspices of IRB-approved protocols. The tissues were minced, and cultured in DMEM medium supplemented with 15% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml gentamicin and 0.25 μg/ml amphotericin B. The cells of passage 3 – 5 were used for invasion assays and siRNA interference assays. All experiments were

approved by the Duke University Institutional Review Board and in accordance with the guidelines outlined by the board.

TGF-β Stimulation

Fibroblasts were stimulated with recombinant human TGF-β1 (R&D Systems). Hyaluronan content in the supernatant was measured using a hyaluronan-specific enzyme-linked immunosorbent assay as described (55). To isolate protein for western blot analysis, the fibroblasts were lysed in RIPA buffer (Sigma) containing phosphatase and protease inhibitor cocktails (Sigma) and centrifuged for 10 min at 10000 rpm. The supernatant was removed and stored at −80 C until further use. Protein content was measured using the BCA assay (Thermo scientific), and 50 μg of total protein per sample was separated on SDS-polyacylamide gels and transferred onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked with 5% BSA in TBST for 1h at room temperature. Immunoblots were probed with primary antibody to phospho-SMAD3 (Cell Signaling, #9520, 1:1000 dilution), PAI-1 (R&D Systems, #AF3828), collagen I (Abcam, #ab21286), α-tubulin (Cell Signaling, #2144, 1:2000 dilution), β-actin, or GAPDH (Cell Signaling, #14C10) at 4°C overnight followed by donkey anti-rabbit or donkey anti-goat secondary antibodies (Jackson ImmunoResearch, 1:10,000 dilution) for 50 min at room temperature.

Fibroblast Migration

Modified Boyden chambers (12-well transwell plate, Costar) were used to measure the chemotaxis of lung fibroblasts to BALF as previously described (53, 56). The BALF was from mice 5 days after administration of 5 U/kg bleomycin. Primary lung fibroblasts (0.5×10^5 cells) in serum-free DMEM were loaded into the top chamber, and BALF diluted in serum-free DMEM containing 0.1% BSA was loaded into the bottom chamber. After 4 hours at 37°C with 5% CO_2, the fibroblasts that migrated across the fibronectin-coated filter were stained with Protocol Hema3 stain set and counted in 5 randomly chosen fields per filter from duplicate filters per sample at 400x magnification.

Fibroblast Invasion

Fibroblasts (0.5×10^5 cells) were loaded into the top chamber of a BioCoat Matrigel Invasion Chamber (BD Biosciences), and DMEM containing 10% FBS was loaded into the bottom chamber. After 24 hours at 37°C with 5% CO_2, the filters were fixed and stained using the Protocol Hema3 stain set. Non-invading cells as well as the basement membrane matrix were removed from the upper side of the filter by gentle scrubbing with a cotton swab. The number of fibroblasts that invaded through the basement membrane was counted in 5 randomly chosen fields per filter from triplicate filters per sample at 400x magnification.

Gene expression analysis

RNA was isolated from fibroblasts using the Qiagen RNeasy kit with added DNAse purification according to manufacturer's adapted protocol using the QiaCube purification rotor. Reverse transcription was performed using the RT^2 First Strand cDNA Synthesis kit (SABiosciences), and 84 genes were assessed by RT-PCR using the Mouse Extracellular Matrix and Adhesion Molecules array (RT^2 Profiler PCR Array PAMM-013; SABiosciences) according to manufacturers instructions using a MyIQ qRT-PCR machine (BioRad). For analysis, the expression level for each gene of interest (GOI) was calculated as 2^{-Ct} followed by normalization to Hprt1, the housekeeping gene (HKG), using the formula $2^{-(Ct\,GOI - Ct\,HKG)}$. Ultimately the fold change in normalized gene expression was calculated by comparing values from knockout fibroblasts purified from bleomycin-treated animals (EXP) to those purified from WT bleomycin-treated animals (CTL) according to the following formula: $2^{-(\Delta Ct\,EXP - \Delta Ct\,CTL)}$. Values were calculated for replicates of three

independent experiments, and p-values were calculated using one-way ANOVA analysis with Bonferoni correction.

siRNA transfection

Fibroblasts isolated from bleomycin-treated mice were transfected with oligos targeting either β-arrestin1 (agccuucugugcugagaac) or β-arrestin2 (ggacggaaaguguucgug), whereas lung fibroblasts isolated from IPF patients were transfected with oligos targeting either β-arrestin1 (ggaccgcaaaguguuugug) or β-arrestin2 (ccaaccucauugaauuuga), using the Transductin siRNA delivery reagent from Integrated DNA Technologies. Briefly, for a 100 mm plate of cells, a reaction mixed 36 μl of 40 μM siRNA in Dharmacon siRNA buffer (300mM KCl, 30mM HEPES-pH 7.5, 1.0mM MgCl2, B-002000-UB-100) with 8 μl of 200 μM Transductin and 164 μl PBS-10% glycerol on ice for 15 min. Cells were washed with serum free media 2x, and then 4 ml of 10% "Q-serum media" was added to each plate. The Q-serum media were prepared as following: 5 ml FBS + 1 ml Source 30Q resin (Amersham Bioscience) was tumbled for 30 minutes at room temperature, was then spun for 5 minutes at 2000rpm, followed by syringe filtration through a 0.22 μm filter. The transfection mixture was diluted 1:5 in 10% Q-serum media and added to the plate for followed by a 6h incubation with final siRNA concentrations at 40 nM. After 6h, the transfection mixture was removed and replaced with 10% FBS media. Cells were analyzed 72 hours post-delivery for invasion capacity or for β-arrestin protein levels by immunobloting probed with an antibody against β-arrestins (A1CT, which recognizes both β-arrestins (10, 19)).

Statistical analysis

Data are presented as mean ± SEM and analyzed by Student's two- tailed t-test and ANOVA followed by Tukey-Kramer honestly significant difference post hoc test. Statistical difference of survival curves was analyzed by log-rank test. A P value < 0.05 is considered statistically significant.

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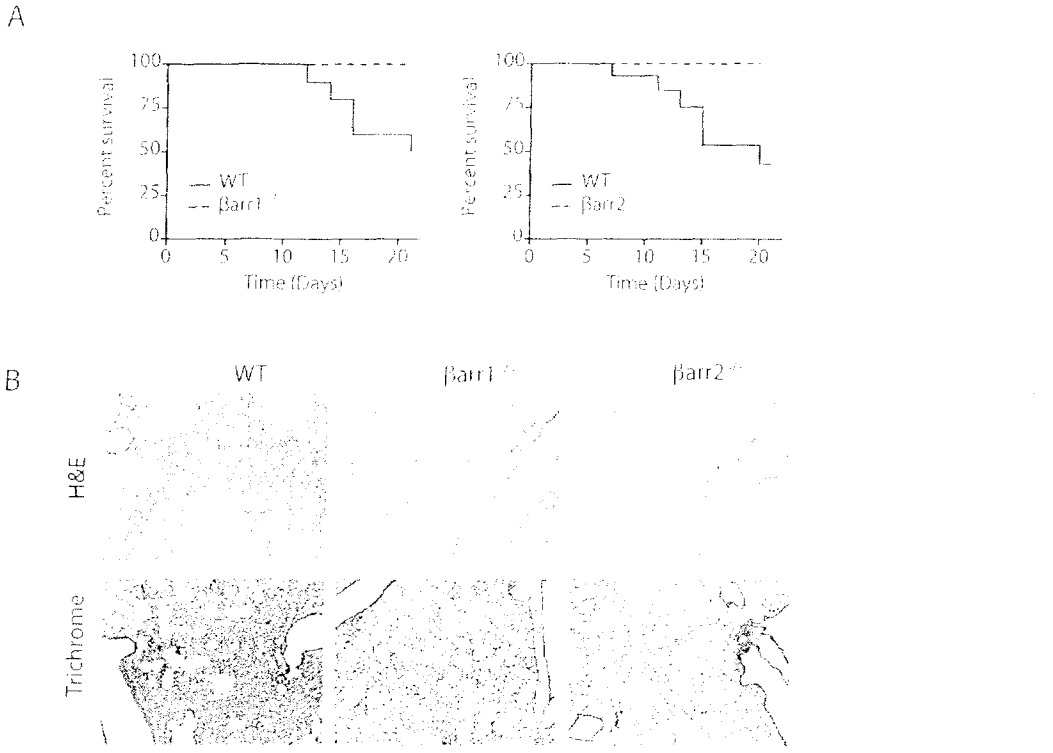

Figure 1.

Loss of either β-arrestin1 or β-arrestin2 protects mice from bleomycin-induced mortality. **(A)** Survival curves for β-arrestin1$^{-/-}$, β-arrestin2$^{-/-}$, and WT mice after 2.5 U/kg bleomycin instillation. n = 10. *P < 0.05. **(B)** Lung sections from the surviving mice at Day 21 were stained with hematoxylin-eosin or Masson's trichrome. Original magnification, 100×.

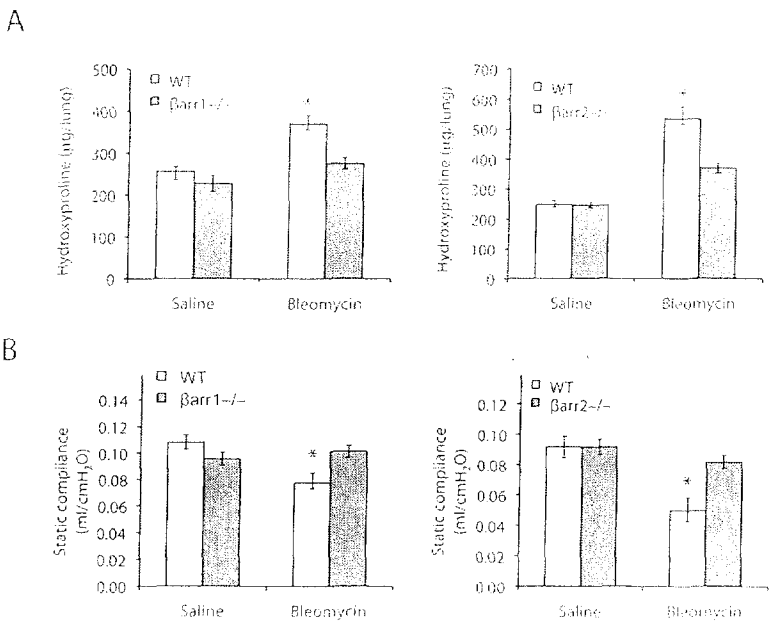

Figure 2.

Loss of either β-arrestin1 or β-arrestin2 protects mice from alterations in lung mechanics and excessive collagen deposition after bleomycin instillation. **(A)** Hydroxyproline content in the lungs of β-arrestin1$^{-/-}$, β-arrestin2$^{-/-}$, and WT mice 21 days after the instillation of 1.25 U/kg bleomycin. **(B)** Lung mechanics were measured in anesthetized, paralyzed, and mechanically ventilated mice 21 days after 1.25 U/kg bleomycin or saline administration. Static compliance was determined by fitting the Salazar-Knowles equation to pressure-volume curves. $n = 10$, $^{*}P < 0.05$ compared to all other groups by ANOVA followed by Tukey-Kramer honestly significant difference post hoc test.

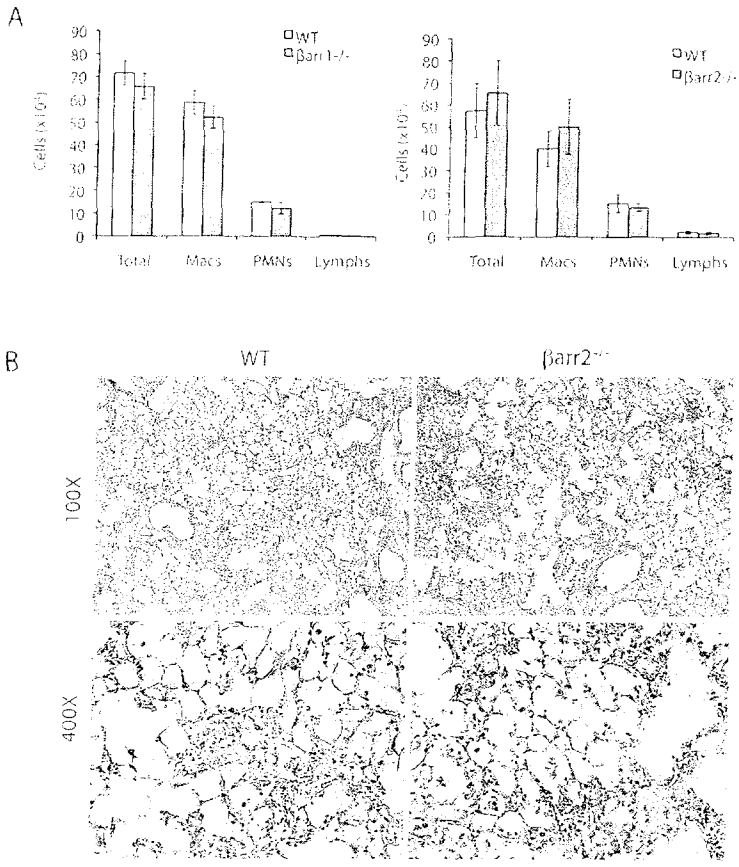

Figure 3.

Inflammatory cell influx is similar in the β-arrestin1$^{-/-}$, β-arrestin2$^{-/-}$, and WT mice. **(A)** Seven days after 1.25 U/kg bleomycin instillation, BALF cells were collected, and total cell counts as well as differential cell counts were determined. n = 5. **(B)** Lung sections from the β-arrestin2$^{-/-}$ and WT mice 7 days after bleomycin treatment were stained with hematoxylin-eosin. Original magnifications are indicated.

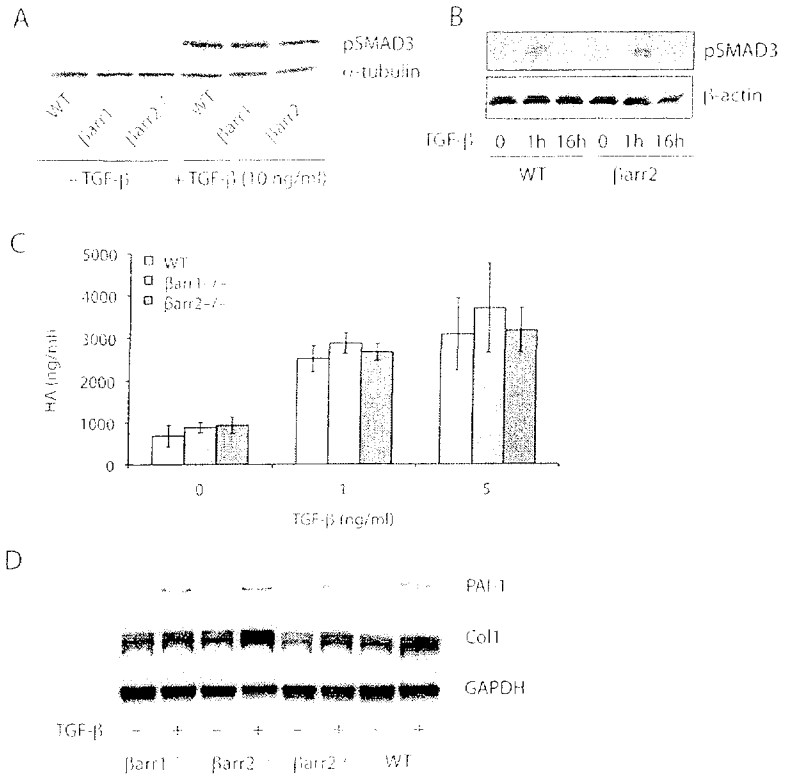

Figure 4.

Primary lung fibroblasts from β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ mice exhibit a normal response to TGF-β stimulation. **(A–B)** Fibroblasts were treated with 10 ng/ml **(A)** or 5 ng/ml **(B)** of TGF-β1 and harvested for protein after 1 **(A–B)** or 16 hours **(B)** of stimulation. Western blot analysis demonstrated a similar amount of phospho-SMAD3 in the WT, β-arrestin1$^{-/-}$, and β-arrestin2$^{-/-}$ fibroblasts. Sample loading was verified by expression of α-tubulin or β-actin. The data are representative of three independent experiments. **(C)** Fibroblasts were stimulated with 1 or 5 ng/ml of TGF-β1 for 24 hours. The cell culture supernatant was removed and analyzed for HA content by ELISA. Data represent mean ± SE of four independent experiments. **(D)** Fibroblasts were treated with 5 ng/ml of TGF-β1 and harvested for protein after 16 hours of stimulation. Western blot analysis demonstrated a similar amount of PAI-1 and collagen I in the WT, β-arrestin1$^{-/-}$, and β-arrestin2$^{-/-}$ fibroblasts. Sample loading was verified by expression of GAPDH.

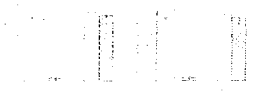

Figure 5.

BALF-induced fibroblast chemotaxis is similar in WT, β-arrestin1$^{-/-}$, and β-arrestin2$^{-/-}$ mice. Primary lung fibroblasts were plated in serum-free DMEM onto modified Boyden chambers with a fibronectin-coated filter. BALF from bleomycin-treated mice was diluted in serum-free DMEM and added to the bottom chamber. After 4 hours, the number of fibroblasts that migrated to the other side of the filter was counted. Data represent mean ± SE of four independent experiments.

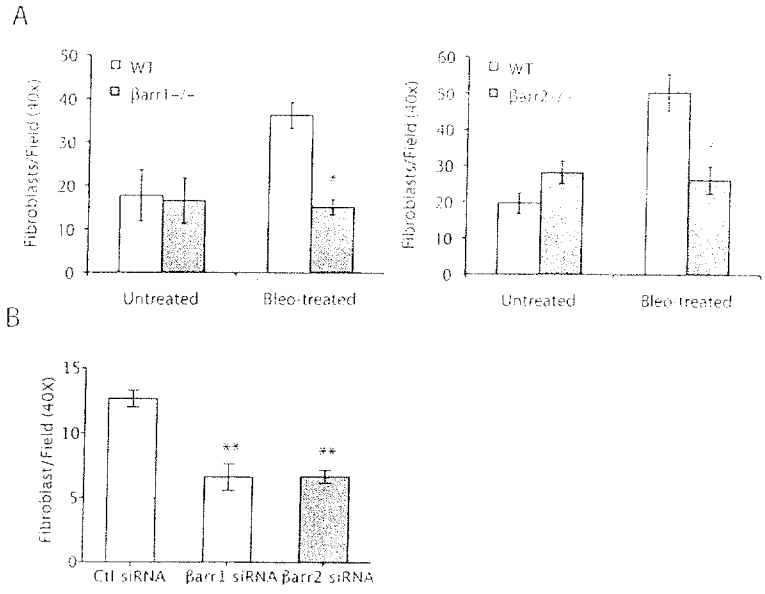

Figure 6.

β-arrestin1 and β-arrestin2 are required for primary lung fibroblast invasion. **(A)** Primary lung fibroblasts were isolated from untreated mice and mice 7 days after bleomycin treatment. After 3 passages, the fibroblasts were loaded onto a matrigel-coated filter, and after 24 hours, the number of fibroblasts that invaded through the matrix was counted. Data represent mean ± SE of four independent experiments. $*P < 0.05$ compared to all other groups by the Tukey-Kramer test for multiple comparisons. **(B)** Primary lung fibroblasts isolated from bleomycin-treated mice were transduced with siRNA specific to β-arrestin1, β-arrestin2, or nonspecific control (Ctl). Invasive capacity of the fibroblasts was assessed with the Matrigel invasion assay. Quantitative analysis of knockdown experiments demonstrated decreased invasion after knockdown of both β-arrestin1 and β-arrestin2. $**P < 0.01$. The experiments were repeated three times.

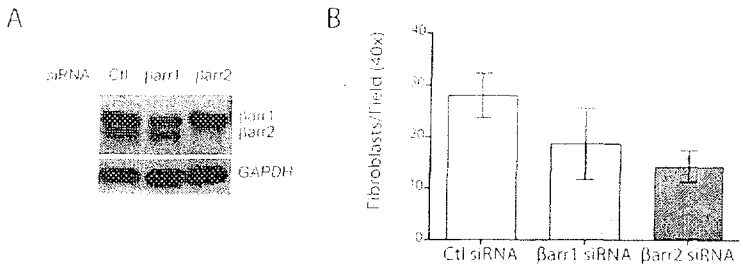

Figure 7.

β-arrestin2 is important in mediating fibroblast invasion of human IPF fibroblasts. **(A)** Primary human lung fibroblasts from IPF patients were transduced with siRNA specific to β-arrestin1, β-arrestin2, or nonspecific control (Ctl) and harvested for protein after 48 hours. Western blot analysis demonstrated a significant reduction of β-arrestin2 and a less robust knockdown of β-arrestin1. Sample loading was verified by blotting of GAPDH. **(B)** Invasive capacity of the fibroblasts was assessed with the Matrigel invasion assay. $^{*}P < 0.05$. β-arrestin2 knockdown was performed on 5 different IPF fibroblast patient samples and β-arrestin1 knockdown on 4 different samples.

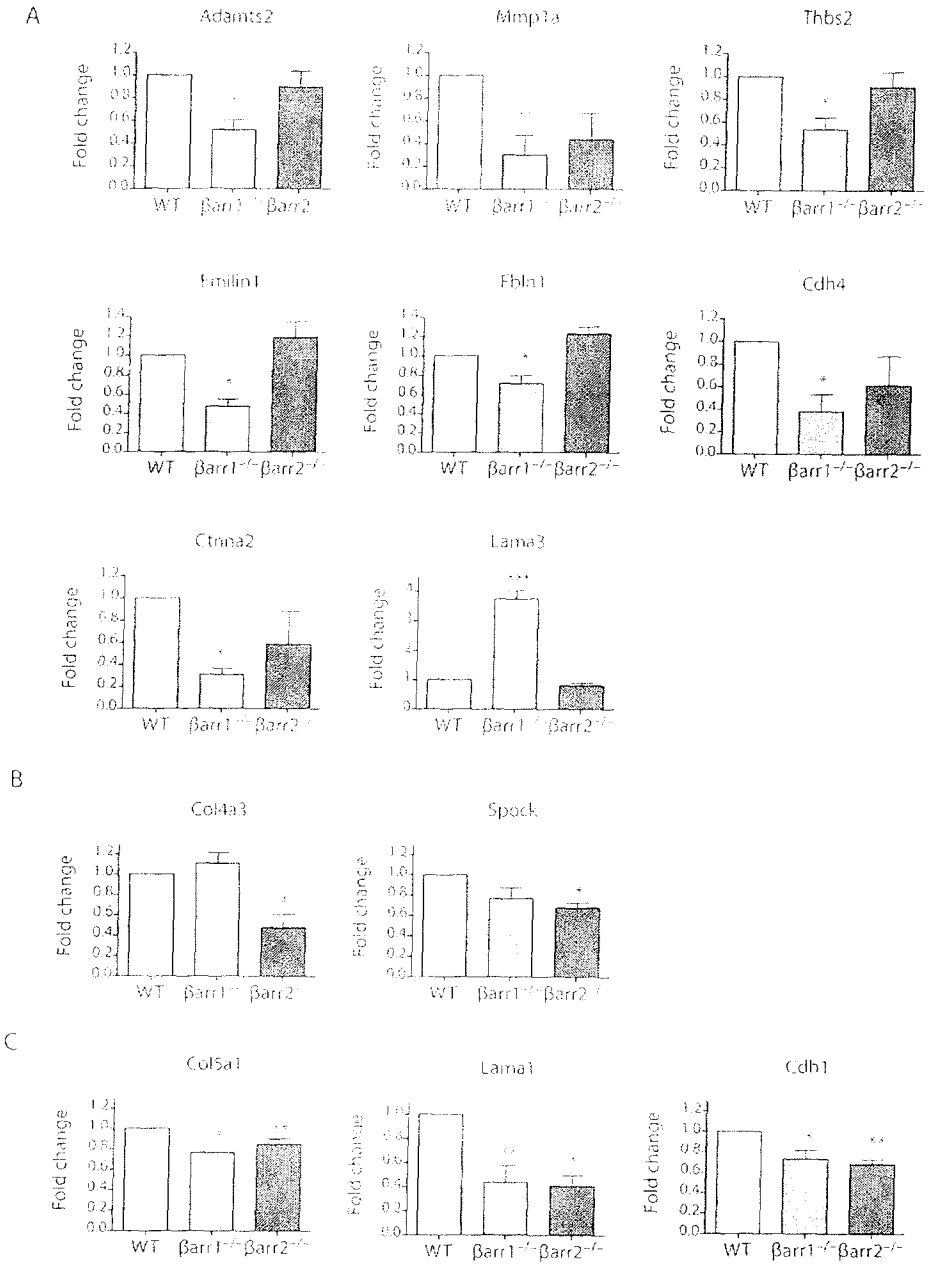

Figure 8.

Loss of β-arrestin1 and β-arrestin2 alters the expression of genes involved in collagen formation and invasion. Using a qRT-PCR array, 84 genes involved in adhesion and extracellular matrix production and degradation were analyzed. **(A)** Genes altered in β-arrestin1$^{-/-}$ primary fibroblasts only. **(B)** Genes altered in β-arrestin2$^{-/-}$ primary fibroblasts only. **(C)** Genes altered in both β-arrestin1$^{-/-}$ and β-arrestin2$^{-/-}$ primary fibroblasts. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as calculated by one-way ANOVA with Bonferoni correction.